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Biological activities of N^6,C8-disubstituted adenosine derivatives as partial agonists at rat brain adenosine A_1 receptors

Anna Lorenzen [a,*], Ana M. Sebastião [b], Andrea Sellink [c], Heidrun Vogt [a], Ulrich Schwabe [a], J. Alexandre Ribeiro [b], Adriaan P. IJzerman [c]

[a] *Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany*
[b] *Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras codex, Portugal*
[c] *Division of Medicinal Chemistry, Leiden–Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

C8-substituted derivatives of the adenosine A_1 receptor-selective agonist N^6-cyclopentyladenosine (CPA) were evaluated as potential partial adenosine A_1 receptor agonists in rat brain. Potencies and efficacies of 8-alkylamino-CPA derivatives were determined in G protein activation assays by their ability to stimulate binding of [^{35}S]guanosine-5′-(γ-thio)triphosphate ([^{35}S]GTPγS) to rat forebrain membranes, by their ability to inhibit forskolin-stimulated adenylate cyclase, and by inhibition of evoked field excitatory postsynaptic potentials (field EPSPs) in hippocampal slices. EC_{50} values around 1 μM were determined for all C8-substituted CPA derivatives. Increase in chain length of the substituent gradually reduced agonist efficacy in [^{35}S]GTPγS binding studies. Only C8-methylamino-, C8-ethylamino- and C8-propylamino-CPA inhibited forskolin-stimulated adenylate cyclase. In contrast, 8-methylamino- and 8-butylamino-CPA were the compounds of highest intrinsic activity in inhibition of field EPSPs in the hippocampus, followed by 8-ethylamino-CPA. 8-Cyclopentylamino-CPA was without effect in this tissue, and the propylamino derivative, when applied cumulatively, caused an inhibition which was smaller the higher the concentration used and the longer the application, which is suggestive of drug-induced desensitization. These data indicate that 8-aminoalkyl-substituted CPA derivatives act as partial agonists on the brain and may serve as valuable tools to dissect adenosine A_1 receptor mediated signal trafficking in various organs. © 1997 Elsevier Science B.V.

Keywords: Adenosine A_1 receptor; G protein; Excitatory postsynaptic potential; Hippocampus; Agonism, partial; Intrinsic activity

1. Introduction

The recently revised ternary complex model of receptor activation (Samama et al., 1993; Bond et al., 1995) predicts that receptors isomerize spontaneously between a basal, inactive conformation R and an active state R^*. The active state R^* is able to activate the G protein and, consequently, to induce effector activation. According to this model, agonists preferentially bind to and stabilize the active conformation R^* of the receptor, whereas antagonists do not differentiate between R and R^*. Inverse agonists would trap receptors in the inactive state R. This model considers receptor coupling to a single G protein subtype. However, signalling through multiple G protein subtypes and effectors has been described for many receptors, including the adenosine A_1 receptor, which can activate G_o, G_{i1}, G_{i2} and G_{i3} (Freissmuth et al., 1991). The concept that a single receptor can adopt several distinct conformational states (i.e., R_1^*, R_2^*, R_3^*, etc.) which induce selective activation of G protein subtypes and, hence, of distinct effector systems, has been termed agonist trafficking (Kenakin, 1995). According to this theory, agonists could induce qualitatively distinct physiological responses through a single receptor subtype by selective activation of effector systems. This possibility of selective activation of only a part of the receptor-induced physiological responses would open a new direction in the search for more advantageous therapeutic agents, which could have reduced side effects, while maintaining the desired therapeutic effect.

The use of adenosine-related therapeutic agents is very limited because of serious side effects, which are due to the ubiquitous localization of adenosine receptors and the wide variety of the effects of adenosine, e.g., on the central nervous system, on the respiratory tract, on the immune

* Corresponding author. Tel.: (49-6221) 548-561; Fax: (49-6221) 548-644; e-mail: anna.lorenzen@urz.uni-heidelberg.de

system and its significant cardiovascular actions. Partial agonists of adenosine receptors might display a lower degree of undesired effects due to decreased efficacy, or – as pointed out above – because of possibly selective induction of the desired effect whilst avoiding side effects. Modifications of the structure of the adenosine molecule which lead to decreased intrinsic activity have been characterized previously. For example, removal of 2′- and/or 3′-hydroxyl groups from the ribose (Taylor et al., 1986; Lohse et al., 1988; Van der Wenden et al., 1995a) and alkylamino substitutions at the C8 position of the purine ring (Van der Wenden et al., 1995b) lead to lower intrinsic activities. Selectivity towards the adenosine A_1 receptor has been achieved by combining these modifications with N^6-cyclopentyl- or N^6-cyclohexyl-substitutions (Van der Wenden et al., 1995a; Mathôt et al., 1995; Roelen et al., 1996).

In the present study, we have investigated potencies and efficacies of C8-aminoalkyl-substituted derivatives of N^6-cyclopentyladenosine (CPA) in G protein activation, as assessed by stimulation of [^{35}S]guanosine-5′-(γ-thio)triphosphate ([^{35}S]GTPγS) binding to rat forebrain membranes. The stable GTP derivative is bound nonselectively to all G protein subtypes upon activation. Hence, receptor stimulation of [^{35}S]GTPγS binding represents a composite response and is useful for an overall estimation of ligand potency and intrinsic activity (Traynor and Nahorski, 1995; Lorenzen et al., 1996). More specifically, induction of a physiological response to adenosine A_1 receptor partial agonists was measured as the inhibition of synaptic transmission in the rat hippocampus. Adenosine A_1 receptor-mediated inhibition of evoked field excitatory postsynaptic potentials (field EPSPs) was monitored and compared to results from G protein activation experiments and inhibition of forskolin-stimulated adenylate cyclase in rat forebrain membranes in order to get insight into the underlying mechanism. The effects of the partial agonists in brain were also compared to efficacies in the cardiovascular system in conscious rats (Roelen et al., 1996) in order to detect compounds with possibly selective effects on either the central nervous system or the cardiovascular system.

2. Materials and methods

[^{35}S]GTPγS (1000–1500 Ci/mmol) and [α-^{32}P]ATP (30 Ci/mmol) were obtained from New England Nuclear (Bad Homburg, Germany). Bovine serum albumin, GTPγS, forskolin and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate) were purchased from Sigma (Deisenhofen, Germany). ATP, cyclic AMP (cAMP), creatine kinase (from rabbit muscle; E.C. 2.7.3.2, 350 U/mg), creatine phosphate (disodium salt), dithiothreitol, GDP and adenosine deaminase (from calf intestine; E.C. 3.5.4.4, 200 U/mg) came from Boehringer-Mannheim (Mannheim, Germany). CPA, DPCPX (1,3-dipropyl-8-cyclopentylxanthine) and Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) were purchased from Research Biochemicals (Köln, Germany). All other chemicals were obtained from standard sources and were of the highest purity commercially available.

2.1. Stimulation of G protein activation in rat brain membranes

Preparations of membranes from rat forebrain were performed as described previously (Lorenzen et al., 1993). Protein content was determined according to Peterson (1977), using bovine serum albumin as standard protein.

Intrinsic activities and potencies of adenosine A_1 receptor agonists were assessed as reported previously (Lorenzen et al., 1996). Briefly, 2 μg of membranes were incubated in a shaking water bath for 90 min in a total volume of 100 μl. Incubations were performed at pH 7.4 and contained 50 mM Tris–HCl, 2 mM triethanolamine, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 10 μM GDP, 0.2 U/ml adenosine deaminase, 0.5% bovine serum albumin and approximately 50 000 cpm (0.2–0.3 nM) [^{35}S]GTPγS. Separation of free from membrane-bound radioligand was done by filtration of the samples over GF/B filters (Whatman, Maidstone, UK), which were washed twice with 4 ml of 50 mM Tris–HCl; pH 7.4, 5 mM $MgCl_2$, containing 0.02% CHAPS.

Adenosine A_1 receptor agonist potencies and intrinsic activities were calculated with SigmaPlot. EC_{50} values were determined from three independent experiments and are given as geometric means with 95% confidence limits. Intrinsic activities of partial agonists are given as relative values with reference to the full agonist CPA set as 100%.

2.2. Inhibition of forskolin-stimulated adenylate cyclase

The ability of 8-aminoalkyl analogues of CPA to inhibit forskolin-stimulated adenylate cyclase in rat forebrain membranes was compared to that of CPA. The adenylate cyclase assay was performed as described by Lohse et al. (1985). The incubation medium contained approximately 250 000 cpm [α-^{32}P]ATP, 100 μM ATP, 10 μM GTP, 100 μM cAMP, 50 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 150 mM NaCl, 500 μM Ro 20-1724, 2 U/ml adenosine deaminase, 0.2 mg bovine serum albumin, 0.13 mg creatine phosphate, 0.04 mg creatine kinase, 1 μM forskolin and 15 μg of membranes in a total volume of 100 μl. Incubations were done for 10 min at 30°C. Dose–response-curves for 8-aminomethyl-CPA and CPA were calculated with SigmaPlot. EC_{50} values determined from three experiments are given as geometric means with 95% confidence limits. For the determination of intrinsic activities of partial adenosine A_1 receptor agonists, all compounds were used at concentrations of $100 \times EC_{50}$ determined in the [^{35}S]GTPγS binding assay. The effects of 8-aminoalkyl-CPA derivatives were compared with the effect of CPA, which was used as the reference full

agonist. Results are given from three independent experiments.

2.3. Electrophysiological recordings from the hippocampus

The experiments were performed on hippocampal slices from male Wistar rats (5 to 6 weeks old). The animals were decapitated under halothane anaesthesia and the hippocampus dissected free into ice-cold bathing solution of the following composition (mM): NaCl 124, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1.25, $NaHCO_3$, 26, glucose 10, gassed with $95\%O_2/5\%CO_2$ (pH ≈ 7.4). Slices were transversely cut 400 μm thick on a McIlwain tissue chopper and allowed to recover for at least 1 h in gassed bathing solution, at room temperature. A slice was then transferred to a 1 ml (plus 2 ml dead volume) recording chamber for submerged slices and continuously superfused with gassed bathing solution at 30.5°C, at a flow rate of 3 ml/min. Drugs were added to this superfusing solution. Except when otherwise indicated, the different concentrations of each drug were applied to the preparations in a cumulative manner. Only one drug was tested in each slice.

Monopolar stimulation (rectangular pulses of 0.1 ms applied once every 10 s) was delivered through a concentric electrode placed on the Schaffer collateral/commissural fibers, in the stratum radiatum near the CA3/CA1 border. Evoked field excitatory postsynaptic potentials (field EPSPs) were recorded through an extracellular microelectrode (4 M NaCl, 2–5 MΩ resistance) placed in the stratum radiatum of the CA1 area. The intensity of the stimulus (120–400 μA) was adjusted to obtain a large field EPSP slope with a minimum population spike contamination (Cunha et al., 1994). Recordings were obtained with an Axoclamp 2B amplifier coupled to a DigiData 1200 interface (Axon Instruments) or through a WPI 750 amplifier coupled to a Tektronix digitalizing oscilloscope. Averages of 8 consecutive responses were continuously monitored on a personal computer with the pclamp (Axon Instruments) software or with the Signal Processing and Display (SPD, Tektronix) software with local modifications. Responses were quantified as the slope of the initial phase of the averaged field EPSPs. The data are expressed as mean ± S.E.M. from n experiments. To calculate the E_{max} (maximal effect), the pD_2 values and the corresponding EC_{50} values, the log concentration–response curve for each drug was fitted to a sigmoid (variable slope and with a constant bottom value of zero) by non-linear regression analysis using the GraphPad Prism software.

3. Results

C8-substituted CPA analogues were tested as putative partial agonists of the adenosine A_1 receptor in studies of G protein activation and by modulation of synaptic transmission. We compared the efficacies and potencies of 8-methylamino-CPA (compound 1), 8-ethylamino-CPA (compound 2), 8-propylamino-CPA (compound 3), 8-butylamino-CPA (compound 4) and 8-cyclopentylamino-CPA (compound 5) to the full agonist CPA with respect to stimulation of [^{35}S]GTPγS binding in rat forebrain membranes and inhibition of field EPSPs in the CA1 area of the hippocampus. These analogues of CPA were also characterized with respect to partial antagonist behavior both in G protein activation and modulation of synaptic transmission.

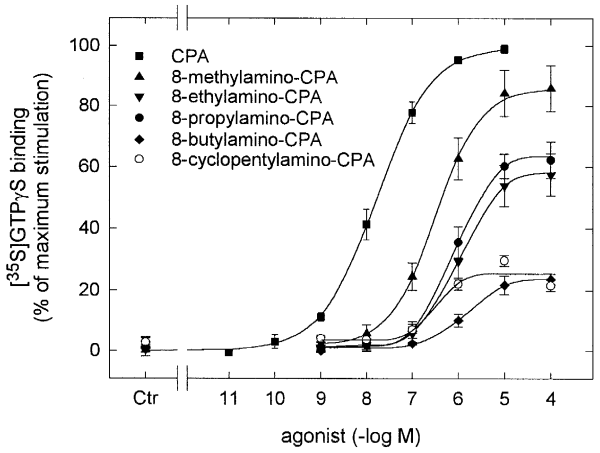

Fig. 1. Stimulation of [^{35}S]GTPγS binding by CPA (■), 8-methylamino-CPA (▲), 8-ethylamino-CPA (▼), 8-propylamino-CPA (·), 8-butylamino-CPA (◆) and 8-cyclopentylamino-CPA (◦) to rat brain membranes. Incubations were performed for 90 min at 25°C as described in Section 2. Data are from three independent experiments and are given as stimulation of G protein activation in relation to the maximal response to the full agonist CPA ± S.E.M. Control binding in the absence of agonists was 0.373 ± 0.026 pmol/mg protein, maximally CPA-stimulated binding values amounted to 0.970 ± 0.038 pmol/mg.

All C8-substituted compounds induced significant stimulation of [^{35}S]GTPγS binding to rat brain membranes and displayed intrinsic activities lower than the parent compound CPA (Fig. 1). The intrinsic activities ranged between 22 and 84% with reference to CPA (Table 1). 8-Methylamino-CPA was the partial agonist with the highest intrinsic activity (84% in comparison to CPA). Increase in the chain length of the C8 substituent gradually reduced the efficacy of the CPA derivatives. 8-Ethylamino- and 8-propylamino-CPA had approximately 60% of the intrinsic activity of the full agonist, whereas 8-butylamino- and 8-cyclopentylamino-CPA displayed efficacies between 20 and 25%.

8-Methylamino-CPA and 8-cyclopentylamino-CPA were the most potent partial agonists to stimulate [^{35}S]GTPγS binding with EC_{50} values of ≈ 300 nM, whereas the ethylamino-, propylamino- and butylamino-CPA derivatives were approximately threefold less potent. All partial agonists had potencies at least twenty times lower than CPA (Table 1). Therefore, it seems clear that

Table 1
Potencies and intrinsic activities of C8-substituted CPA derivatives in G protein activation

	C8 substitution	EC_{50} (nM) (95% c.l.)	Efficacy (% of CPA)
CPA		16.7 (9.9–28.1)	100
1	NH–CH_3	320 (235–435)	83.8 ± 6.3
2	NH–CH_2–CH_3	1030 (743–1420)	57.7 ± 4.4
3	NH–$(CH_2)_2$–CH_3	838 (647–1080)	62.4 ± 4.3
4	NH–$(CH_2)_3$–CH_3	1420 (737–2740)	23.0 ± 0.8
5	NH–cyclopentyl	304 (212–437)	22.0 ± 0.7

G protein activation was assessed by stimulation of [^{35}S]GTPγS binding to rat forebrain membranes. Membranes (2 μg) were incubated for 90 min with CPA or C8-substituted CPA derivatives as described in Section 2. EC_{50} values and 95% confidence limits (95% c.l.) were calculated from three experiments for each agonist. Agonist efficacies were calculated from the difference between basal and maximally stimulated binding and are given as percentage of stimulation ± S.E.M. relative to the full agonist CPA (100%).

C8 substitutions do not only affect intrinsic activity, but also the potency of adenosine A_1 receptor ligands. However, as can be seen from Table 1, these characteristics are not necessarily changed in a parallel fashion by different C8 substitutions. The correlation between potency and intrinsic activity of the compounds investigated was not significant. Therefore, it seems possible to synthesize C8-substituted partial agonists of the adenosine A_1 receptor with low intrinsic activity and relatively high affinity.

C8-aminoalkyl-CPA derivatives also acted as inhibitors of G protein activation induced by the full agonist CPA (Fig. 2). When [^{35}S]GTPγS binding was measured in the presence of increasing concentrations of the putative partial agonists and a maximally stimulating concentration of CPA, it was observed that the partial agonists inhibited guanine nucleotide binding in response to the full agonist. The inhibition of the CPA effect obtained in the presence of high concentrations of partial agonists was also a partial inhibition, because levels of [^{35}S]GTPγS binding in the presence of CPA (1 μM) and maximum concentrations of the respective partial agonist were identical to [^{35}S]GTPγS binding in the presence of a maximum concentration of the partial agonist alone, but not to control binding in the absence of adenosine A_1 receptor ligands (Fig. 2).

The ability of the compounds investigated to activate G proteins was compared with their ability to inhibit forskolin-activated adenylate cyclase in forebrain membranes. Both CPA and 8-methylamino-CPA inhibited forskolin-stimulated adenylate cyclase in a concentration-dependent way. The maximum inhibition induced by CPA was approximately twofold greater than that of 8-methylamino-CPA (Fig. 3). EC_{50} values were 279 (95% confidence limits: 189–414) nM for CPA and 4880 (2580–9230) nM for 8-methylamino-CPA. The intrinsic activities of putative partial agonists in relation to CPA were estimated at single concentrations of all compounds of $100 \times EC_{50}$ derived from [^{35}S]GTPγS dose–response-curves. CPA inhibited 27 ± 3% of forskolin-stimulated enzyme activity. Of the partial agonists tested, only 8-methylamino-CPA, 8-ethylamino-CPA and 8-propylamino-CPA were able to inhibit adenylate cyclase, but showed much lower intrinsic activities than CPA (Table 2). 8-Butylamino- and 8-cyclopentylamino-CPA did not affect forskolin-stimulated adenylate cyclase.

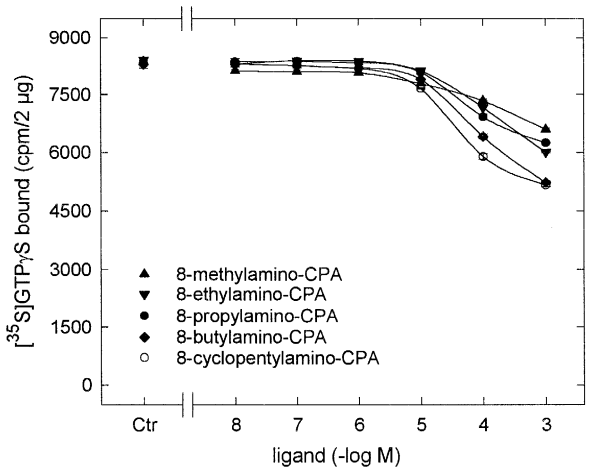

Fig. 2. Inhibition of CPA-induced G protein activation by 8-aminoalkyl-CPA analogues. Membranes were incubated in the presence of 1 μM CPA and in the presence of increasing concentrations (10 nM–1 mM) of putative partial agonists (▲, 8-methylamino-CPA; ▼, 8-ethylamino-CPA; ·, 8-propylamino-CPA; ◆, 8-butylamino-CPA; and ◦, 8-cyclopentylamino-CPA). [^{35}S]GTPγS binding was measured after an incubation period of 90 min at 25°C as described in Section 2. Results ± S.E.M. from three independent experiments are shown. Control binding in the absence of adenosine receptor ligands was 3360 ± 33 cpm. [^{35}S]GTPγS binding in the presence of 100 μM of the 8-aminoalkyl-CPA derivatives in the absence of CPA amounted to 7338 ± 9 cpm (8-methylamino-CPA), 6205 ± 27 cpm (8-ethylamino-CPA), 6137 ± 40 cpm (8-propylamino-CPA), 4429 ± 26 cpm (8-butylamino-CPA), and 4296 ± 67 cpm (8-cyclopentylamino-CPA).

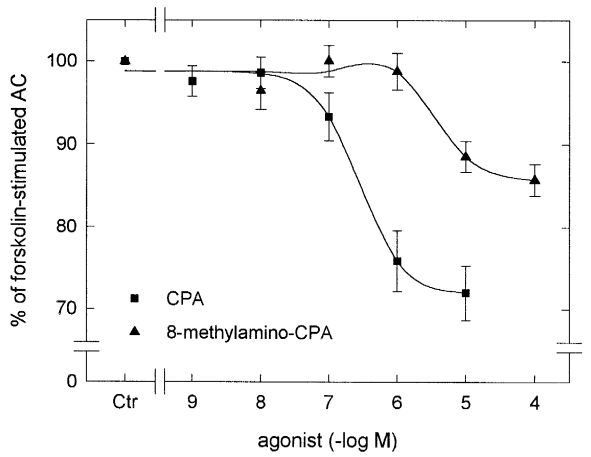

Fig. 3. Inhibition of forskolin-stimulated adenylate cyclase (AC) by CPA and 8-methylamino-CPA. 15 μg of rat forebrain membranes were incubated with increasing concentrations of CPA or 8-methylamino-CPA in the presence of 1 μM forskolin for 10 min at 30°C as outlined in Section 2. The figure shows dose–response-curves from three experiments. Data are given as means ± S.E.M.

C8-substituted CPA derivatives were further investigated in electrophysiological studies. Fig. 4A illustrates the inhibition of field EPSPs caused by one of these analogues, 8-butylamino-CPA. It can be observed that 8-butylamino-CPA (1–30 μM) caused a reversible and concentration-dependent decrease of the slope of the field EPSPs, as well as a decrease of the amplitude of the field EPSPs (Fig. 4A, lower panel), which was quantitatively similar to the decrease in the field EPSP slope.

The inhibitory effect of 8-butylamino-CPA on field EPSPs was antagonized by the selective adenosine A_1 receptor antagonist, DPCPX. Thus, in one slice 8-butylamino-CPA (30 μM) caused a 96% decrease of field EPSP slope in the absence of DPCPX, whereas it was virtually devoid of effect on field EPSPs when applied to the slice 60 min after starting perfusion of DPCPX (50 nM) (Fig. 4B). In the same experiment it was observed that two successive applications of the same concentration of 8-butylamino-CPA caused a similar inhibition of the field EPSP slope. The ability of DPCPX (50 nM) to antagonize the inhibitory effect of 8-methylamino-CPA (30 μM) on field EPSPs was also tested, and it was observed that the agonist decreased the field EPSP slope by 84% in the absence of the adenosine A_1 receptor antagonist, and by only 3% when applied to the same slice 60 min after perfusion with DPCPX. A similar protocol was used for the antagonism by DPCPX (50 nM) of the effect of 8-ethylamino-CPA (30 μM), which decreased the field EPSP slope by 15% in the absence of DPCPX and by 0% in its presence.

The full effect of each concentration of 8-butylamino-CPA was observed within 15–25 min after starting its perfusion and was washed out within 20 min after starting its removal from the bath (Fig. 4A, upper panel). Most of the other CPA analogues tested, as already described for CPA (Cunha et al., 1994), had a slower time course of the effect. Thus, in the experiments described in this paper, the full effect of each concentration of CPA (cf. Fig. 7) and of 8-methylamino-CPA were observed within 30–35 min after starting the perfusion, and that of 8-ethylamino-CPA was observed within 20–30 min. It then appears that an increase in the carbon chain length progressively decreases the time needed for development of the full drug effect. Nevertheless, and as a precaution to avoid underestimation of the effects of the drugs due to non-equilibrium conditions, the usual procedure was to monitor continuously the averaged field EPSPs and to perfuse each drug concentration for about 45 min. The decrease of the field EPSP slope caused by each drug concentration was usually calculated by averaging the responses over the last 8 min of its application, i.e., by averaging 6 consecutive averaged field EPSPs. The value obtained was then compared with that obtained by a similar procedure during 8 min in precontrol conditions before drug application.

In Fig. 5 are illustrated the log concentration–response curves for the effects of CPA, 8-methylamino-CPA, 8-ethylamino-CPA, 8-butylamino-CPA and 8-cyclopentylamino-CPA. All these compounds, except 8-cyclopentylamino-CPA, decreased in a reversible and concentration-dependent manner the slope of the field EPSPs, the CPA analogues being markedly less potent than CPA. 8-Cyclopentylamino-CPA (30–100 μM) was virtually devoid of effect on either field EPSP slope or field EPSP amplitude.

The calculated affinity (pD_2 and corresponding EC_{50} values) and the efficacy (E_{max}) of CPA and CPA analogues to inhibit synaptic transmission in the CA1 area of the hippocampus were calculated from the fitting of the log concentration–response curves (see Fig. 5) to a sigmoid, and the results obtained are summarized in Table 2. It is evident that the EC_{50} values for 8-methylamino-CPA, 8-ethylamino-CPA and 8-butylamino-CPA were within the same order of magnitude, and approximately two orders of magnitude higher than the EC_{50} value for CPA. 8-Ethylamino-CPA had a significantly lower efficacy ($P < 0.05$) than 8-methylamino-CPA, which also had a significantly lower efficacy ($P < 0.05$) than CPA. The calculated efficacy of 8-butylamino-CPA was between that of 8-methylamino-CPA and CPA, and significantly ($P < 0.05$) greater than that of 8-ethylamino-CPA.

Another CPA analogue tested, 8-propylamino-CPA gave quite unexpected results in relation to its ability to inhibit synaptic transmission in the CA1 area of the hippocampus. When a single concentration of this compound was tested in each slice, it was observed that the higher the concentration the greater was the inhibition of the field EPSP slope (1 μM: 13%, $n = 1$; 3 μM: 29%, $n = 1$; 10 μM: 37%, $n = 1$). However, when this drug was applied at increasing concentrations (1–100 μM) in a cumulative way to the

Table 2
Potencies and efficacies of 8-alkylamino-CPA derivatives to inhibit synaptic transmission in the hippocampus

	C8 substitution	pD_2	EC_{50} (nM) (95% c.l.)	E_{max} (%)
CPA		7.890 ± 0.028	12.9 (11.3–14.7)	92 ± 3.3
1	NH–CH_3	5.995 ± 0.073	1010 (708–1440)	75 ± 3.8 *
2	NH–CH_2–CH_3	5.888 ± 0.114	1290 (727–2300)	31 ± 2.5 *
4	NH–$(CH_2)_3$–CH_3	5.557 ± 0.272	2770 (731–10 500)	81 ± 14

The ability of CPA and CPA derivatives to inhibit synaptic transmission was assessed by their ability to decrease the slope of field EPSPs recorded from the CA1 area of rat hippocampal slices upon stimulation of the Schaffer collaterals. The values in the table were calculated by nonlinear regression analysis of the data shown in Fig. 5 as described in Fig. 2.

* $P < 0.05$ as compared with CPA.

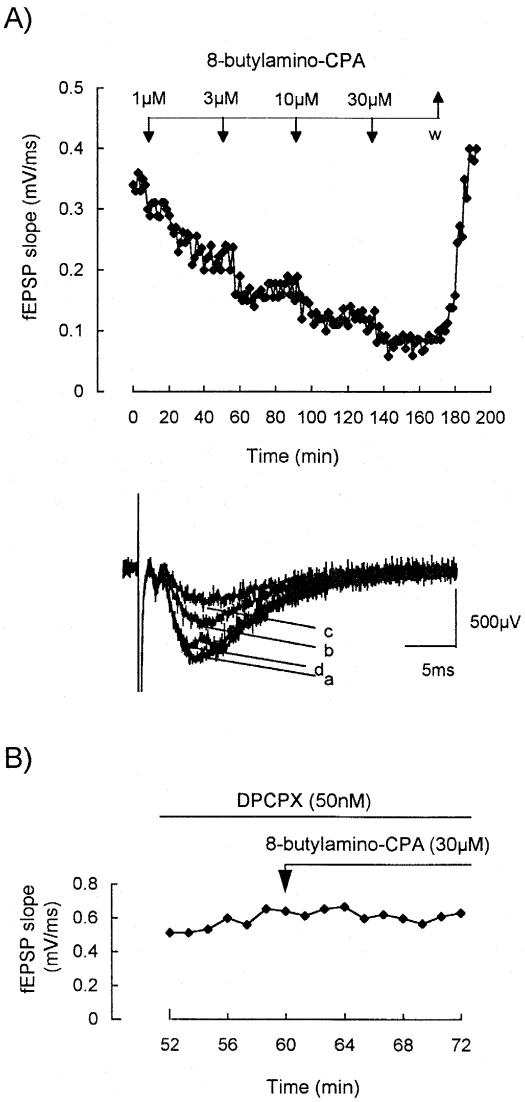

Fig. 4. Effect of 8-butylamino-CPA (A) and antagonism of this effect by the adenosine A_1 receptor antagonist DPCPX (B) on averaged field EPSPs recorded from the CA1 area of rat hippocampal slices upon stimulation of the Schaffer collaterals. In (A), the upper panel represents the timecourse of the effect of 8-butylamino-CPA. Each point corresponds, in ordinates, to the slope of the average of 8 consecutive field EPSPs, and in the abscissae to the start of averaging. The beginning of the superfusion of each concentration of 8-butylamino-CPA and of the washout (w) is indicated by the arrows. The lower panel shows superimposed recordings of averaged field EPSPs recorded from the same slice before drug application (a), during the full effect of 3 μM (b) and 30 μM (c) 8-butylamino-CPA, and upon the agonist washout (d). The field EPSPs are preceded by the synaptic voley and the stimulus artifact. In (B) is shown the absence of effect of 8-butylamino-CPA (30 μM) when applied to another slice 60 min after starting the perfusion of DPCPX (50 nM). In the same slice, but before perfusion of DPCPX, 8-butylamino-CPA had decreased field EPSP slope by 96%.

slices, the higher the concentration the smaller was the observed decrease in field EPSP slope (Fig. 6). The concentration of 100 μM even caused a small increase (17 ± 2%) of the field EPSP slope, as compared with its value before applying the drug.

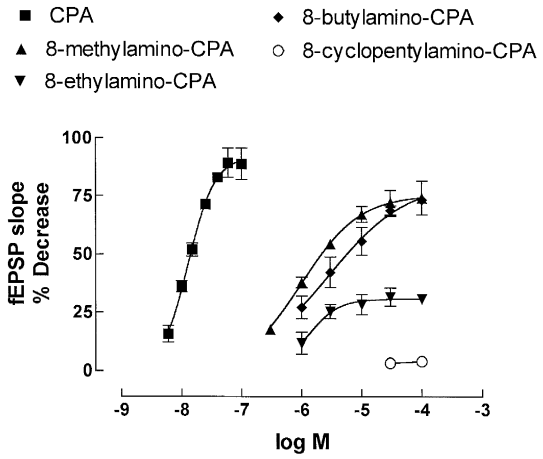

Fig. 5. Concentration–response curves for the effects of CPA (■), 8-methylamino-CPA (▲), 8-ethylamino-CPA (▼), 8-butylamino-CPA (◆) and 8-cyclopentylamino-CPA (○) on the slope of field EPSPs recorded from the CA1 area of rat hippocampal slices upon stimulation of the Schaffer collaterals. In the ordinates, 0% is the field EPSP slope before drug application, and 100% represents a complete inhibition of field EPSPs. The data for each drug, except for 8-cyclopentylamino-CPA, were obtained in four to five slices taken from different animals. 8-Cyclopentylamino-CPA was tested in one slice. The averaged slope of the field EPSPs before drug application was 0.404 ± 0.030 mV/ms, and it recovered to 108 ± 4% of this value upon washout of the agonists.

The possibility that 8-ethylamino-CPA, which has low (but measurable) intrinsic activity as inhibitor of synaptic transmission in the hippocampus (Tables 2 and 3), could

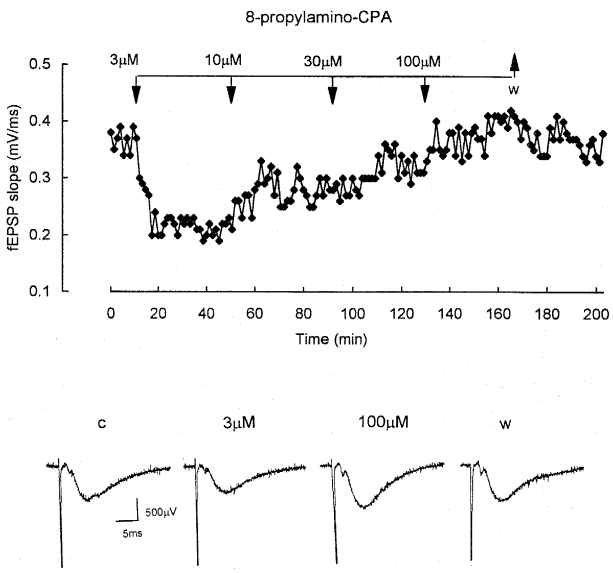

Fig. 6. Time course of the effect of cumulative applications of increasing concentrations of 8-propylamino-CPA on averaged field EPSPs recorded from the CA1 area of a rat hippocampal slice. The beginning of the superfusion of each concentration of 8-propylamino-CPA and of the washout (w) is indicated by the arrows. The lower panel shows recordings of averaged field EPSPs in control conditions before drug application (c), during the full effect of 3 μM and of 100 μM 8-propylamino-CPA, and upon the agonist washout (w). For further details see legend to Fig. 4. Note that the inhibitory effect of 8-propylamino-CPA was smaller the higher the concentration of this agonist, 100 μM even causing a small excitatory effect.

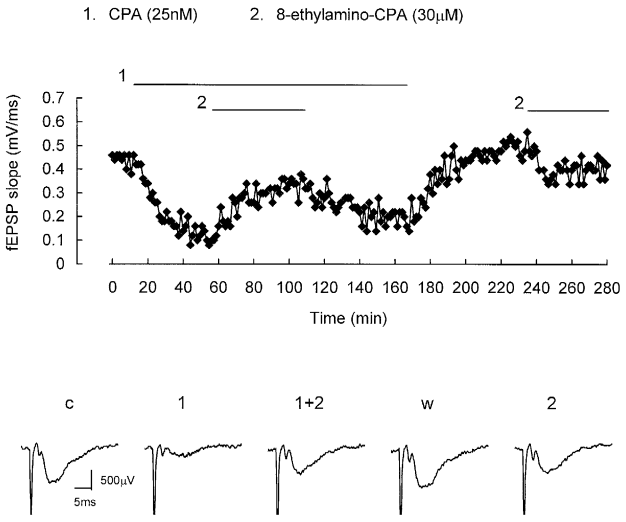

Fig. 7. Antagonism of the effect of CPA on field EPSPs by the partial agonist 8-ethylamino-CPA. The upper panel represents the time course of the effects, and the lower panel shows recordings of averaged field EPSPs under the conditions indicated above each trace and immediately before changeover of solutions. The presence of CPA (25 nM) is labeled by 1 and the presence of 8-ethylamino-CPA (30 μM) is labeled by 2; c refers to pre-control conditions and w to washout. For further details see legend to Fig. 4.

behave as an antagonist of the effect of the full agonist CPA was tested in one experiment (Fig. 7). The initial application of CPA (25 nM) caused, as expected, a decrease (78%) in the field EPSP slope. Keeping the concentration of CPA constant, the perfusion of 8-ethylamino-CPA (30 μM) was started, and the field EPSP slope progressively increased up to a level which was only 23% smaller than the pre-control field EPSP slope, i.e., before drug application. This antagonism (71%) caused by 8-ethylamino-CPA of the effect of CPA was reversible since upon removal of the partial agonist from the bath the field EPSP slope decreased again, provided that CPA was present in the bath. At the end of the experiment, and after washing out CPA, 8-ethylamino-CPA (30 μM) applied alone decreased the field EPSP slope by 21% (Fig. 7).

4. Discussion

From the present results we conclude that 8-aminoalkyl CPA derivatives have lower potency and efficacy than CPA in G protein activation in forebrain and in inhibition of synaptic transmission in the CA1 hippocampal area. The apparent potency of CPA to stimulate [^{35}S]GTPγS binding and to inhibit the field EPSPs in the CA1 area of the hippocampus, with an EC_{50} value in the low nanomolar range, is in accordance with its ability to activate inhibitory hippocampal adenosine receptors, which are of the A_1 subtype (Sebastião et al., 1990). The EC_{50} values of the 8-aminoalkyl derivatives to inhibit field EPSPs in the hippocampus and to activate G proteins in forebrain membranes are in good correlation to their K_i values for cerebral cortical adenosine A_1 receptors in the presence of GTP (Roelen et al., 1996). The 8-cycloalkyl derivative tested, 8-cyclopentylamino-CPA, had an efficacy near zero in the hippocampus and was also the partial agonist of lowest efficacy to stimulate [^{35}S]GTPγS incorporation into adenosine A_1 receptor-coupled G proteins. This is in agreement with the small GTP shift obtained in the adenosine A_1 receptor binding experiments (Roelen et al., 1996). Besides a low efficacy, an additional criteria to define partial agonists is their ability to antagonize the effects of full agonists (Jasper and Insel, 1992). Accordingly, it was observed that C8-substituted CPA derivatives, which acted as partial agonists in the [^{35}S]GTPγS binding assay and in synaptic transmission, also antagonized CPA-mediated adenosine A_1 receptor activation when assessed either as G protein activation or as inhibition of field EPSPs. That the 8-aminoalkyl-CPA derivatives are acting on adenosine A_1 receptors is reinforced by the observation that their effects were fully prevented by the adenosine A_1 receptor antagonist DPCPX.

However, there were significant differences in the ability of 8-aminoalkyl CPA derivatives to activate adenosine A_1 receptors when assessed as G protein or second messenger activation or as inhibition of field EPSPs. These differences were noted in intrinsic activities rather than in apparent affinities. The intrinsic activities determined in

Table 3
Intrinsic activities of C8-substituted CPA derivatives in comparison to the full agonist CPA in vitro and in vivo

	C8 substitution	GTP shift [a]	[^{35}S]GTPγS binding	AC inhibition	Field EPSP inhibition	Heart rate reduction [a]	MAP decrease [a]
CPA		6.0	100	100	100	100	100
1	NH–CH_3	3.8	84	38	82	63	82
2	NH–CH_2–CH_3	2.8	58	49	34	46	39
3	NH–$(CH_2)_2$–CH_3	3.0	62	20	(des)	37	25
4	NH–$(CH_2)_3$–CH_3	2.3	23	0	88	15	18
5	NH–cyclopentyl	1.2	22	0	0	4	5

Intrinsic activities of CPA and derivatives were estimated by measurement of GTP shifts (i.e., ratios between the affinities determined in the absence of 1 mM GTP), G protein activation and inhibition of adenylate cyclase (AC) in rat brain membranes, and inhibition of the slope of field EPSPs in rat hippocampal slices in vitro as well as by reduction of heart rate and mean arterial pressure (MAP) in vivo in normotensive conscious rats. Intrinsic activities (except GTP shift) are given as percentage of response in relation to the full agonist CPA set as 100%. (des): rapid desensitization.

[a] Data from Roelen et al., 1996.

the present study are only in partial agreement with the intrinsic activities of C8-substituted CPA derivatives in the reduction of heart rate and decrease of mean arterial pressure in vivo (Table 3). By increasing the chain length in the C8 position of the CPA molecule, a gradual decrease in the ability to activate G proteins as well as to reduce heart rate and mean arterial blood pressure was observed, as predicted by decreasing GTP shifts (Roelen et al., 1996). In the hippocampus, the increase in carbon chain length from a methyl to an ethyl group induced a more marked decrease in the efficacy to inhibit the field EPSPs. 8-Cyclopentylamino-CPA was virtually ineffective in the inhibition of synaptic transmission in the hippocampus. The greatest discrepancy was noted for 8-butylamino-CPA. 8-Butylamino-CPA had a significantly greater efficacy than 8-ethylamino-CPA and even slightly greater than 8-methylamino-CPA in the inhibition of synaptic transmission in the hippocampus. This high efficacy ($\approx 80\%$) cannot be attributed to a putative ability of this compound to inhibit other inhibitory receptors in this brain area, since the effect of a near maximal concentration of 8-butylamino-CPA was fully antagonized by the selective adenosine A_1 receptor antagonist DPCPX. The high efficacy of 8-butylamino-CPA might be of interest in the design of adenosine A_1 receptor agonists with a selective action in the hippocampus, since it only marginally affected heart rate and blood pressure (Roelen et al., 1996).

The underlying mechanisms for selective actions of partial adenosine A_1 receptor agonists on different target organs have not been investigated. It is known that the efficacy of agonists is influenced by receptor density and coupling to different G proteins and effectors. Higher receptor densities have been shown to increase the intrinsic activities of β-adrenergic agonists (Whaley et al., 1994; MacEwan et al., 1995). Partial agonists of the 5-HT_{1A}-receptor display higher intrinsic activities in cells with higher levels of receptor expression (Hoyer and Boddeke, 1993). Accordingly, partial agonists of the adenosine A_1 receptor could activate target organs depending on receptor reserve. Alternatively, different G protein subtypes could mediate their physiological effects. Because partial agonists do not necessarily activate receptor-coupled G proteins to identical degrees, it seems conceivable that partial agonists could exert organ-selective actions, depending on the G protein coupled to the receptor in the respective tissue (Kenakin, 1995). Selective activation of G protein subtypes has been shown for 5-HT_{1A}-receptors (Gettys et al., 1994) and α_1 and α_2 adrenoceptors (Eason et al., 1994; Perez et al., 1996). Adenosine A_1 receptors may interact with G proteins of the G_i and G_o subtype (Munshi et al., 1991; Freissmuth et al., 1991). In the present study, G protein activation has been measured by stimulation of [^{35}S]GTPγS binding, which does not differentiate between G protein subtypes. Inhibition of adenylate cyclase by adenosine A_1 receptors is mediated by G proteins of the G_i subtype. Determination of the ability of C8-alkylamino-CPA derivatives demonstrated that intrinsic activities of these compounds to inhibit $G_{i\alpha}$ activation, as assessed by adenylate cyclase inhibition, were smaller than their intrinsic efficacies in [^{35}S]GTPγS binding studies (Table 3). In addition, efficacies in adenylate cyclase inhibition did not correlate with efficacies in field EPSP inhibition (Table 3). We therefore assume that these processes are mediated via different G protein subtypes, which is in good agreement with the notion that the inhibition of transmitter release by adenosine A_1 receptors in hippocampus proceeds independently of cAMP levels (Dunwiddie and Fredholm, 1985). Since G_o has been identified as the G protein which links A_1 receptors to inhibition of L-type Ca^{2+} channels (Sweeney and Dolphin, 1995), further investigations could address the influence of partial adenosine A_1 receptor agonists on this G protein subtype and this effector system.

Another interesting difference between the results obtained for inhibition of field EPSPs in the hippocampus and in G protein activation was observed with 8-propylamino-CPA. This compound showed moderate affinity ($\approx 0.8\mu M$) and efficacy ($\approx 60\%$) when tested in [^{35}S]GTPγS binding studies. However, in relation to its ability to inhibit field EPSPs in the hippocampus, the results obtained with the cumulative and non-cumulative applications of this compound are highly suggestive of its ability to quickly desensitize hippocampal adenosine A_1 receptors. Desensitization of hippocampal adenosine A_1 receptors by 8-propylamino-CPA might explain the smaller effect of a higher concentration of the drug when applied cumulatively after a smaller concentration, as well as the excitatory effect of the highest concentration of 8-propylamino-CPA tested, which was probably due to blockade of the tonic inhibitory action of endogenous adenosine on adenosine A_1 receptors in the hippocampus (see Sebastião et al., 1990; Cunha et al., 1996). Whether the putative ability of 8-propylamino-CPA to desensitize adenosine A_1 receptors in the hippocampus will prove of interest for the design of adenosine derivatives with moderate inhibitory actions on the cardiovascular system and virtually no inhibitory neuronal actions, awaits further investigation.

In summary, as predicted (Roelen et al., 1996), partial agonism of adenosine A_1 receptor could induce selectivity of effects in different tissues. This is the case for 8-butylamino-CPA which was highly effective for the adenosine A_1 receptors from one brain area, the hippocampus, while having negligible adenosine A_1-mediated cardiovascular actions. Conversely, 8-propylamino-CPA might be virtually devoid of sustained inhibitory actions in the CNS, due to receptor-induced desensitization, while still keeping some cardiovascular inhibitory properties. These differences might be related to differences in receptor reserve, in the nature of the transducing system, in the degree of receptor-transducing system coupling, or to other differences in the receptor environment and might prove useful in the design of tissue-selective adenosine-related therapeutic compounds.

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