Naunyn-Schmiedeberg's Arch Pharmacol (1989) 340:679–683

2-Chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) – a high affinity agonist radioligand for A_1 adenosine receptors

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Summary. The tritiated analogue of 2-chloro-N^6-cyclopentyladenosine (CCPA), an adenosine derivative with subnanomolar affinity and a 10000-fold selectivity for A_1 adenosine receptors, has been examined as a new agonist radioligand. [^{3}H]CCPA was prepared with a specific radioactivity of 1.58 TBq/mmol (43 Ci/mmol) and bound in a reversible manner to A_1 receptors from rat brain membranes with a high affinity K_D-value of 0.2 nmol/l. In the presence of GTP a K_D-value of 13 nmol/l was determined for the low affinity state for agonist binding. Competition of several adenosine receptor agonists and antagonists for [^{3}H]CCPA binding to rat brain membranes confirmed binding to an A_1 receptor. Solubilized A_1 receptors bound [^{3}H]CCPA with similar affinity for the high affinity state. At solubilized receptors a reduced association rate was observed in the presence of $MgCl_2$, as has been shown for the agonist [^{3}H]N^6-phenylisopropyladenosine ([^{3}H]PIA). [^{3}H]CCPA was also used for detection of A_1 receptors in rat cardio myocyte membranes, a tissue with a very low receptor density. A K_D-value of 0.4 nmol/l and a B_{max}-value of 16 fmol/mg protein was determined in these membranes. In human platelet membranes no specific binding of [^{3}H]CCPA was measured at concentrations up to 400 nmol/l, indicating that A_2 receptors did not bind [^{3}H]CCPA. Based on the subnanomolar affinity and the high selectivity for A_1 receptors [^{3}H]CCPA proved to be a useful agonist radioligand for characterization of A_1 adenosine receptors also in tissues with very low receptor density.

Key words: Adenosine receptors – Radioligands – Agonists

Introduction

Adenosine acts at cell surface receptors as a modulator of many physiological functions (for review see Gerlach and Becker 1987). Adenosine receptor subtypes have been defined on the basis of pharmacological and biochemical studies. The A_1 receptor inhibits adenylate cyclase via the inhibitory guanine nucleotide binding protein G_i, while the A_2 receptor mediates a stimulation of cyclase via G_s (van Calker et al. 1978; Londos et al. 1980). A_1 adenosine receptors are not only coupled to adenylate cyclase but also modulate K^+-channels (Kurachi et al. 1986), guanylate cyclase (Kurtz 1987) and Ca^{2+}-mobilization (Arend et al. 1988). By means of photoaffinity labelling the A_1 receptor protein has been shown to be a glycoprotein with a molecular weight of 35000 (Klotz et al. 1985; Klotz and Lohse 1986).

Several agonist radioligands are available for the characterization of A_1 adenosine receptors including [^{3}H]CHA (Bruns et al. 1980), [^{3}H]PIA (Schwabe and Trost 1980) and [^{3}H]CPA (Williams et al. 1986). These radioligands have successfully been used to label A_1 adenosine receptors in tissues with high receptor density, e.g. brain membranes or fat cell membranes. Detection of A_1 receptors in tissues like the myocardium with only very low receptor density has been possible only with iodinated agonists (Lohse et al. 1985; Linden et al. 1985; Martens et al. 1987) or with the high-affinity antagonist [^{3}H]DPCPX (Lohse et al. 1987). Tritiated agonists, owing to the low specific radioactivity compared to iodinated radioligands, failed to label receptors in myocardial membranes. We now report the development of a tritiated analogue of CCPA, an agonist with high affinity in the subnanomolar range and an unusually high selectivity for A_1 receptors (Lohse et al. 1988a). [^{3}H]CCPA is a radioligand which proved to be useful in the characterization of A_1 receptors in tissues with low receptor density.

Material and methods

Materials. [^{3}H]PIA was purchased from Du Pont-New England Nuclear (Dreieich, FRG) and [^{3}H]DPCPX from Amersham Buchler (Braunschweig, FRG). GTP was obtained from Boehringer Mannheim (Mannheim, FRG), CHAPS and PEI were from Sigma (Deisenhofen, FRG). All other chemicals were of highest purity available.

Synthesis of CCPA, CCCPA and tritiated analogues. The synthesis of CCPA was started from 2,6-dichloro-9-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)purine and cyclopentylamine with a previously described procedure (Cristalli et al. 1986; Lohse et al. 1988a).

2-Chloro-N^6-cyclopentenyladenosine as a precursor for [^{3}H]CCPA was prepared as follows. To 0.7 g (1.56 mmol) of 2,6-dichloro-9-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)purine

Abbreviations. CHA, N^6-cyclohexyladenosine; CPA, N^6-cyclopentyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CCCPA, 2-chloro-5′-chloro-5′-deoxy-N^6-cyclopentyladenosine; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, N-ethylcarboxamidoadenosine; PEI, polyethylenimine; PIA, N^6-phenylisopropyladenosine

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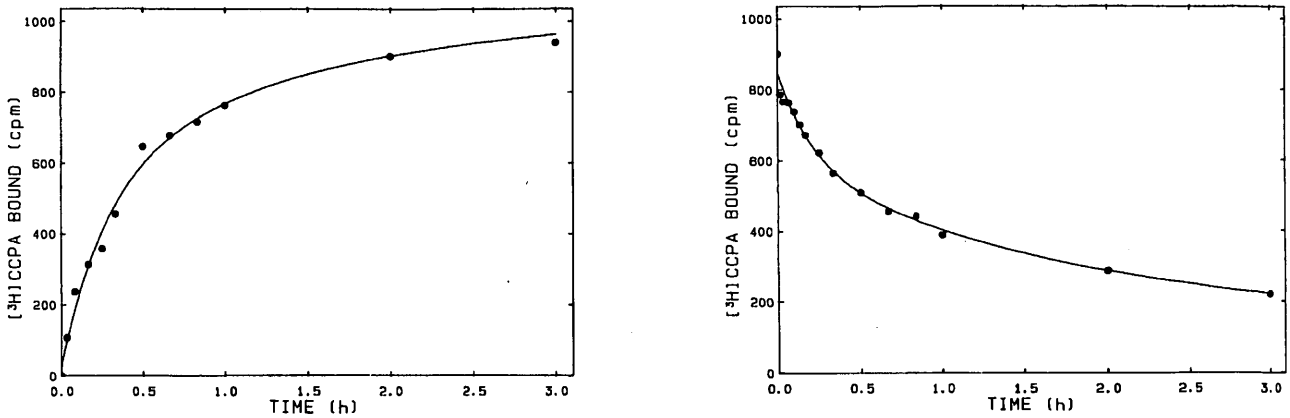

Fig. 1. Association and dissociation curves for [^{3}H]CCPA binding. Shown is specific binding of [^{3}H]CCPA at a final concentration of 0.5 nmol/l to A_1 receptors of rat brain membranes from a single experiment, which was repeated with the same results. Binding equilibrium at room temperature was reached in approximately 2 h (*left panel*). Addition of theophylline at a final concentration of 1 mmol/l induced dissociation of [^{3}H]CCPA in a biphasic manner (*right panel*). The rapidly dissociating binding represented 38% with $t_{1/2}$ 6.7 min and 62% of the binding was slowly dissociating with $t_{1/2}$ 81 min

in 30 ml of methanol 1.6 g (20 mmol) 3-aminocyclopentene was added and the mixture was stirred at room temperature overnight. The solution was concentrated *in vacuo* and the residue was purified by flash chromatography eluting with chloroform-methanol (96:4) to give 0.35 g (0.94 mmol) of light yellow crystals, m.p. 103–106°C; ^{1}H NMR (Me_2SO-d_6) δ 1.66–2.66 (large m, 4H, CH_2-4 and CH_2-5 cyclopentenyl), 3.13–3.43 (m, 1H, 3H cyclopentenyl), 3.64 (m, 2H, CH_2-5′), 3.99 (m, 1H, H4′), 4.18 (m, 1H, H3′), 4.55 (m, 1H, H2′), 5.81 (m, 1H, H-1 cyclopentenyl), 5.88 (d, J = 6 Hz, 1H, H-1′), 5.98 (m, 1H, H-2 cyclopentenyl), 8.35 (d, J = 7.5 Hz, 1H, NH), 8.43 (s, 1H, H-8). Anal. ($C_{15}H_{18}ClN_5O_4$)C,H,N; FW 367.80. The 5′-modification of CCPA, which leads to CCCPA, was introduced following the procedure described by Taylor et al. (1986) with some modifications. To 4 ml of hexamethylphosphoramide were added 1.26 g (10.6 mmol) of thionyl chloride and 0.38 g (1.03 mmol) of CCPA under nitrogen. The mixture was stirred at room temperature for 12 h and then neutralized with concentrated ammonium hydroxide and extracted with ethyl acetate. The extracts were washed twice with water, dried and concentrated *in vacuo*. The residue was purified by flash chromatography (silica gel 60; 230–400 mesh ASTM, Merck) eluting with chloroform-methanol (99:1) to give 0.35 g (0.9 mmol) of a white solid: mp 105–107°C; ^{1}H NMR (Me_2SO-d_6) δ 1.51–2.03 (large m, 8H, H cyclopentyl), 3.90 (m, 2H, CH_2-5′), 4.11 (m, 1H, H-4′), 4.18 (m, 1H, H-3′), 4.43 (m, 1H, H cyclopentyl), 4.66 (m, 1H, H-2′), 5.87 (d, J = 5.8 Hz, 1H, H-1′), 8.37 (s, 1H, H-8), 8.40 (d, 1H, NH). Anal. ($C_{15}H_{19}Cl_2N_5O_3$)C,H,N; FW 388.26.

^{1}H NMR spectra were obtained with a Varian EM-390 90-MHz spectrometer. All exchangeable protons were confirmed by addition of D_2O. Microanalytical results are indicated by atomic symbols and are within ± 0.4% of theoretical values.

Catalytic reduction of 2-chloro-N^6-cyclopentenyladenosine to [^{3}H]CCPA was done by Du Pont de Nemours Inc, Boston, USA. The radioligand with a specific radioactivity of 1.58 TBq/mmol (42.8 Ci/mmol) will be available from Du Pont, NEN products. [^{3}H]CCCPA was prepared from [^{3}H]CCPA by the procedure described above for CCCPA.

Preparation of membranes and solubilized receptors. Rat brain membranes and solubilized A_1 receptors were prepared as described earlier (Klotz et al. 1986). The EDTA-washing step was omitted when membranes were used for radioligand binding. For solubilization of membranes 1% CHAPS in H_2O was used.

Membranes from rat cardiomyocytes were prepared as described by Martens et al. (1987).

Human platelet membranes and solubilized A_2 receptors were prepared according to Lohse et al. (1988b).

Radioligand binding. Radioligand binding to membrane-bound receptors was performed at room temperature for 3 h according to Lohse et al. (1987). Binding to solubilized receptors was done at 12°C for about 20 h as described earlier (Klotz et al. 1986). [^{3}H]CCPA was used at a final concentration of 0.5 nmol/l in a total volume of 250 µl (500 µl in saturation experiments). The protein content was 30 to 50 µg for brain membranes and 230 to 250 µg for myocyte membranes. Nonspecific binding of [^{3}H]CCPA was determined in the presence of 1 mmol/l theophylline. Data were analyzed by nonlinear curve-fitting with the program SCTFIT as described (Lohse et al. 1987). Saturation and displacement curves were fitted according to a one-site model, when a two-site model did not improve the fit significantly ($p \leq 0.001$).

Results

Kinetic experiments on rat brain membranes demonstrated that binding equilibrium with 0.5 nmol/l [^{3}H]CCPA was achieved within 2 h at 25°C (Fig. 1). Dissociation of [^{3}H]CCPA was induced with 1 mmol/l theophylline and showed that the radioligand bound in a reversible manner to A_1 receptors (Fig. 1). Saturation experiments gave a K_D-value of 0.21 nmol/l for the high-affinity state (Fig. 2). Binding to the low-affinity state was not reliably detected under these conditions. Nonspecific binding amounted to about 4% of total binding at K_D. In the presence of 100 µmol/l GTP, [^{3}H]CCPA bound to the low-affinity state of the receptors, and a K_D-value of 13.4 nmol/l was determined (Fig. 3).

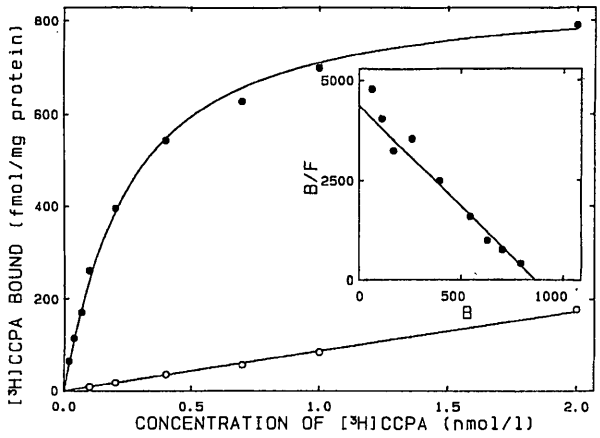

Fig. 2. Saturation of [^{3}H]CCPA binding to rat brain membranes. Data are from a representative experiment and are given as specific (●) and nonspecific (○) binding. A K_D-value of 0.2 nmol/l and a B_{max}-value of 860 fmol/mg protein was determined by non-linear curve fitting. The inset shows the Scatchard plot from the data

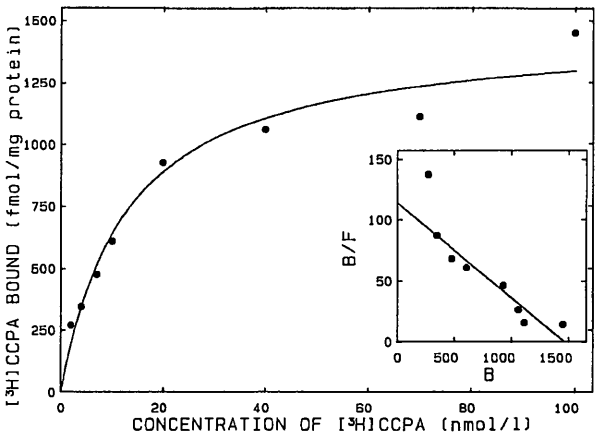

Fig. 3. Saturation of [^{3}H]CCPA binding to rat brain membranes in the presence of 100 µmol/l GTP. Non-linear curve fitting gave a K_D-value of 13.4 nmol/l and a B_{max}-value of 1480 fmol/mg protein. The inset shows the Scatchard plot from the data

Table 1. Pharmacological profile of [^{3}H]CCPA binding to rat brain membranes. Data are means of 2–3 experiments

Compound	K_i (nmol/l)
CCPA	0.19
CCCPA	0.36
R-PIA	0.91
NECA	2.8
S-PIA	18.5
DPCPX	0.3
Theophylline	5750

High and low affinity binding was also measured for the nonradioactive CCPA. Competition for [^{3}H]DPCPX binding to rat brain membranes resulted in a biphasic displacement curve with K_i-values of 0.24 and 18.5 nmol/l for the high-affinity and low-affinity states, respectively (Fig. 4). GTP shifted the curve to the right and from the monophasic curve a K_i-value of 55.6 nmol/l was calculated.

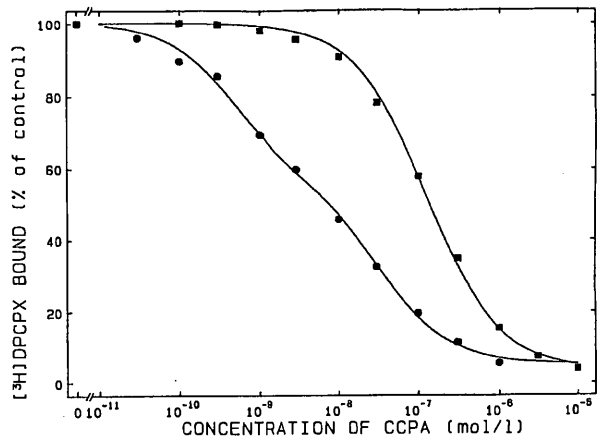

Fig. 4. Competition for [^{3}H]DPCPX binding to A_1 adenosine receptors of rat brain membranes by CCPA. Binding of [^{3}H]DPCPX was measured in the absence (●) and presence of 100 µmol/l GTP (■). Data are given as percentage of total binding of [^{3}H]DPCPX in the absence of CCPA. Control binding (100%) amounted to 220 and 280 fmol/mg protein in the absence and presence of GTP, respectively. In the absence of GTP the curve was best fitted according to a two-side model and K_i-values of 0.24 and 18.5 nmol/l were calculated. In the presence of GTP only one affinity state with a K_i-value of 55.6 nmol/l was detected

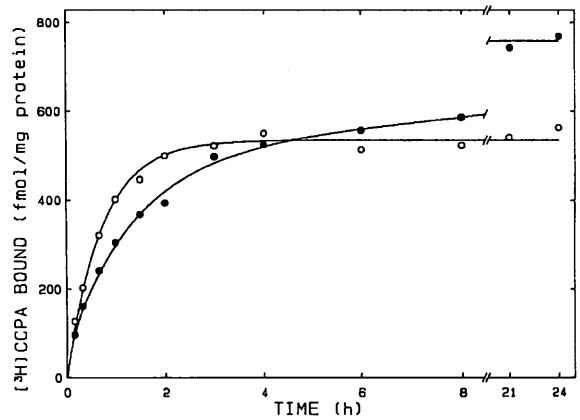

Fig. 5. Association time course of [^{3}H]CCPA to solubilized A_1 receptors. The association was measured at 12°C in the absence (○) and presence (●) of 100 µmol/l $MgCl_2$

Competition by several agonists and antagonists for [^{3}H]CCPA binding was measured to confirm that [^{3}H]CCPA binds to an A_1 adenosine receptor. The K_i-values exhibit the typical pharmacological profile for A_1 receptors with the marked stereoselectivity for the PIA enantiomeres and high affinity binding of DPCPX (Table 1).

The replacement of the 5'-hydroxyl by a 5'-chloro substituent at N^6-substituted adenosine derivatives has been reported to enhance A_1 receptor selectivity (Taylor et al. 1986). This additional modification, which leads to CCCPA, did not further increase A_1 affinity compared to CCPA (Table 1). Binding data for CCCPA at A_2 receptors were also very similar to the data for CCPA (not shown). A K_D-value of 0.29 nmol/l was determined for [^{3}H]CCCPA at A_1 receptors of rat brain membranes (Table 2).

The time course of association of [^{3}H]CCPA to solubilized A_1 receptors from rat brain membranes was mea-

Table 2. Comparison of agonist radioligands for A_1 adenosine receptors of rat brain membranes

Radioligand	K_D (nmol/l)	Specific activity (Ci/mmol)
[^{3}H]PIA	1.4[a]	49
[^{3}H]CPA	0.5[b]	46
[^{3}H]CCCPA	0.3	43
[^{3}H]CCPA	0.2	43

[a] Data from Lohse et al. (1984)
[b] Data from Williams et al. (1986)

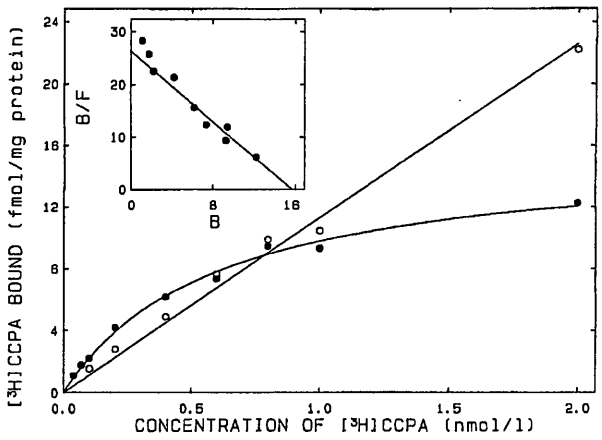

Fig. 6. Saturation of [^{3}H]CCPA binding to rat myocyte membranes. Data are from a representative experiment and are given as specific (●) and nonspecific (○) binding. Non-linear curve fitting gave a K_D-value of 0.59 nmol/l and a B_{max}-value of 16 fmol/mg protein. The inset shows the Scatchard plot from the data

Table 3. K_D-values for [^{3}H]CCPA at A_1 receptors from different rat tissues. The data are geometric means with 95% confidence limits from 3–4 separate experiments

Tissue	K_D (nmol/l)
Brain	
membranes	0.21 (0.19–0.23)
Solubilized receptors (control)	0.24 (0.14–0.39)
Solubilized receptors ($MgCl_2$)	0.15 (0.05–0.42)
Myocyte membranes	0.43 (0.21–0.88)

sured in the absence and presence of 100 µmol/l $MgCl_2$ (Fig. 5). At 12°C, binding equilibrium was reached after about 3 h under control conditions, while in the presence of $MgCl_2$ the association was markedly slowed down. Saturation analysis gave high-affinity K_D-values of 0.24 nmol/l and 0.15 nmol/l in the absence and presence of $MgCl_2$, respectively (Table 3).

Saturation experiments were also performed on membranes of rat ventricular myocytes (Fig. 6). From the data shown in Table 3 a K_D-value of 0.43 nmol/l and a B_{max}-value of 16 fmol/mg was calculated. Nonspecific binding amounted to about 40% of total binding at K_D. Thus, [^{3}H]CCPA proved to be a suitable agonist to label A_1 receptors in tissues with very low receptor density.

Binding of [^{3}H]CCPA was tested in human platelets to examine whether this radioligand retained the high A_1 selectivity of the nonradioactive compound. Both with platelet membranes and solubilized A_2 receptors no specific binding of [^{3}H]CCPA was observed at concentrations up to 400 nmol/l (not shown).

Discussion

Different modifications at the N^6-position of adenosine led in the past to agonists with high affinity and selectivity for A_1 adenosine receptors. In particular, the N^6-cyclopentyl analogue of adenosine, CPA, is a potent and A_1-selective compound (Moos et al. 1985). In a series of 1-deaza analogues of adenosine we have recently shown that a 2-chloro-substitution of 1-deaza-CPA enhanced A_1 selectivity (Cristalli et al. 1988). This observation led subsequently to the synthesis of CCPA with an almost 10000-fold selectivity for the A_1 receptor and a subnanomolar affinity (Lohse et al. 1988a).

The high affinity and selectivity of CCPA prompted us to develop a new radioligand based on this compound. [^{3}H]CCPA exhibits subnanomolar affinity for A_1 receptors with a K_D-value of 0.2 nmol/l. GTP shifted the receptors to a low-affinity state with a K_D-value of 13 nmol/l, demonstrating that binding of [^{3}H]CCPA is GTP sensitive in a manner characteristic for agonists at G protein-coupled receptors. Competition of several agonists and antagonists for [^{3}H]CCPA binding showed the pharmacological profile for an A_1 adenosine receptor. In particular the stereoselectivity for the PIA enantiomeres and the high affinity binding of DPCPX demonstrated that [^{3}H]CCPA labels A_1 receptors. It has been shown that the association rate of [^{3}H]PIA at solubilized A_1 receptors is markedly attenuated by Mg^{2+}-ions (Klotz et al. 1986). This was also observed for [^{3}H]CCPA suggesting that this radioligand possesses all the characteristics of an A_1 receptor agonist.

Trivedi et al. (1989) described recently [^{3}H](S)-ENBA ([^{3}H]1R,2S,4S-2-endo-norbornyladenosine, specific radioactivity 29.3 Ci/mmol) as a radioligand with subnanomolar affinity (K_D = 0.33 nmol/l). Compared to this radioligand [^{3}H]CCPA has first of all a higher specific radioactivity and exhibits in addition a slightly higher affinity at A_1 receptors.

In analogy to the 2-chloro modification of CPA a 5′-chloro-5′-deoxy modification of N^6-substituted adenosine derivatives was reported to also increase A_1 selectivity (Taylor et al. 1986; Trivedi et al. 1989). We therefore synthesized CCCPA as a derivative with both modifications. No additional increase in A_1 selectivity or A_1 affinity occurred. Likewise, [^{3}H]CCCPA was not superior to [^{3}H]CCPA.

Detection of A_1 receptors in tissues with very low receptor density has been successful so far only with [^{3}H]DPCPX (Lohse et al. 1987) or with radioiodinated agonists (Linden et al. 1985; Lohse et al. 1985). The high affinity of [^{3}H]CCPA suggested that this radioligand might be advantageous for labelling of A_1 receptors in tissues like rat heart, where only 18 fmol receptors/mg protein have been found with ^{125}I-HPIA (Martens et al. 1987). Saturation experiments proved that [^{3}H]CCPA is an agonist radioligand which can be used instead of radioiodinated agonists for the detection of A_1 receptors in tissues with low receptor density.

No binding of [^{3}H]CCPA, at concentrations up to 400 nmol/l, was observed to both membrane-bound or

solubilized A_2 receptors from human platelets. It can be roughly estimated that the K_D-value of [^{3}H]CCPA at A_2 receptors should be higher than 4 μmol/l. Thus, the about 10000-fold A_1-selectivity of CCPA seemed to be preserved for the tritiated compound.

In summary, it is concluded that [^{3}H]CCPA is a new agonist radioligand with high selectivity for A_1 receptors, exhibiting virtually no affinity for A_2 receptors. The subnanomolar affinity for A_1 receptors makes [^{3}H]CCPA an important tool for the characterization of receptors in different tissues, in particular for tissues with very low receptor density.

Acknowledgement. We gratefully acknowledge the expert technical assistance of Ms. Heidrun Vogt. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schw 83/13-4) and the European Science Foundation.

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Received July 10, 1989/Accepted August 18, 1989