2-Chloro-N⁶-[³H]cyclopentyladenosine ([³H]CCPA) —
a high affinity agonist radioligand for A₁ adenosine receptors

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Summary. The tritiated analogue of 2-chloro-N⁶-cyclo­pentlyadenosine (CCPA), an adenosine derivative with sub­nanomolar affinity and a 10000-fold selectivity for A₁ adenosine receptors, has been examined as a new agonist radioligand. [³H]CCPA was prepared with a specific radio­activity of 1.58 TBq/mmol (43 Ci/mmol) and bound in a reversible manner to A₁ receptors from rat brain membranes with a high affinity K_p-value of 0.2 nmol/l. In the presence of GTP a K_p-value of 12 nmol/l was determined for the low affinity state for agonist binding. Competition of several adenosine receptor agonists and antagonists for [³H]CCPA binding to rat brain membranes confirmed binding to an A₁ receptor. Solubilized A₁ receptors bound [³H]CCPA with similar affinity for the high affinity state. At solubilized receptors a reduced association rate was observed in the presence of MgCl₂, as has been shown for the agonist [³H]N⁶-phenylisopropyladenosine ([³H]PIA). [³H]CCPA was also used for detection of A₁ receptors in rat cardio­myocyte membranes, a tissue with a very low receptor den­sity. A K_p-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 [³H]CCPA was measured at concentrations up to 400 nmol/l, indicating that A₂ receptors did not bind [³H]CCPA. Based on the sub­nanomolar affinity and the high selectivity for A₁ receptors [³H]CCPA proved to be a useful agonist radioligand for characterization of A₁ 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 de­

Abbreviations. CHA, N⁶-cyclohexyladenosine; CPA, N⁶-cy­clopentlyadenosine; CCPA, 2-chloro-N⁶-cyclopen­tyladenosine; CCCPA, 2-chloro-5'-chloro-5'-deoxy-N⁶-cyclopentyladenosine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesul­fonate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, N-ethylcarboxamidoadenosine; PEI, polyethyl­enimine; PIA, N⁶-phenylisopropyladenosine

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Material and methods

Materials. [³H]PIA was purchased from Du Pont-New England Nuclear (Dreieich, FRG) and [³H]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-N⁶-cyclopentyladenosine as a precursor for [³H]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

Reference

Gupta et al. 1978; Londos et al. 1980. A₁ adenosine receptors are not only coupled to adenylyl cyclase but also modulate K⁺ channels (Kurachi et al. 1986), guanylate cyclase (Kurtz 1987) and Ca²⁺-mobilization (Arend et al. 1988). By means of photoaffinity labelling the A₁ receptor protein has been shown to be a glycoprotein with a molecular weight of 35 000 (Klotz et al. 1985; Klotz and Lohse 1986).

Several agonist radioligands are available for the char­acterization of A₁ adenosine receptors including [³H]CHA (Brus et al. 1980). [³H]PIA (Schwabe and Trost 1980) and [³H]CPA (Williams et al. 1986). These radioligands have successfully been used to label A₁ adenosine receptors in tissues with high receptor density, e.g. brain membranes or fat cell membranes. Detection of A₁ 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 high­affinity antagonist [³H]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 A₁ receptors (Lohse et al. 1988a). [³H]CCPA is a radioligand which proved to be useful in the characterization of A₁ receptors in tissues with low receptor density.
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 \textit{in vacuo} and the residue was purified by flash chromatography eluting with chloroform-methanol (96:4) to give light yellow crystals, mp. 103 - 106°C. $^1$H NMR (Me$_2$SO-d$_6$) $\delta$ 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$_2$-3'), 3.99 (m, 1H, H$^4$), 4.18 (m, 1H, H$^5$), 4.55 (m, 1H, H$^2$), 5.81 (m, 1H, H-1 cyclopentenyl), 5.88 (d, J = 6 Hz, 1H, H-1'), 5.98 (m, 1H, H-2 cyclopentenyl), 8.37 (s, 1H, H-8), 8.43 (s, 1H, H-9), 10.56 (s, 1H, HN). Anal. (C$_{15}$H$_{19}$Cl$_2$N$_5$O$_3$)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 of CCPA, which leads to CCCPA, was introduced following

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$_2$O 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. (1988a).

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). [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 [H]CCCPA was determined in the presence of 1 nmol/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 [H]CCCPA was achieved within 2 h at 25°C (Fig. 1). Dissociation of [H]CCCPA 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 $K_a$-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 $K_p$. In the presence of 100 µmol/l GTP, [H]CCCPA bound to the low-affinity state of the receptors, and a $K_p$-value of 13.4 nmol/l was determined (Fig. 3).
Fig. 2. Saturation of [3H]CCPA binding to rat brain membranes. Data are from a representative experiment and are given as specific (●) and nonspecific (○) binding. A Kᵦ-value of 0.2 nmol/l and a B₅₀-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 [3H]CCPA binding to rat brain membranes in the presence of 100 μmol/l GTP. Non-linear curve fitting gave a Kᵦ-value of 13.4 nmol/l and a B₅₀-value of 1480 fmol/mg protein. The inset shows the Scatchard plot from the data.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵦ (nmol/l)</th>
</tr>
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<tbody>
<tr>
<td>CCPA</td>
<td>0.19</td>
</tr>
<tr>
<td>CCCPA</td>
<td>0.36</td>
</tr>
<tr>
<td>R-PIA</td>
<td>0.91</td>
</tr>
<tr>
<td>NECA</td>
<td>2.8</td>
</tr>
<tr>
<td>S-PIA</td>
<td>18.5</td>
</tr>
<tr>
<td>DPCPX</td>
<td>0.3</td>
</tr>
<tr>
<td>Theophylline</td>
<td>5750</td>
</tr>
</tbody>
</table>

High and low affinity binding was also measured for the nonradioactive CCPA. Competition for [3H]DPCPX binding to rat brain membranes resulted in a biphasic displacement curve with Kᵦ-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 Kᵦ-value of 55.6 nmol/l was calculated.

Fig. 4. Competition for [3H]DPCPX binding to A₁ adenosine receptors of rat brain membranes by CCPA. Binding of [3H]DPCPX was measured in the absence (●) and presence of 100 μmol/l GTP (○). Data are given as percentage of total binding of [3H]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 Kᵦ-values of 0.24 and 18.5 nmol/l were calculated. In the presence of GTP only one affinity state with a Kᵦ-value of 55.6 nmol/l was detected.

Fig. 5. Association time course of [3H]CCPA to solubilized A₁ receptors. The association was measured at 12°C in the absence (○) and presence (●) of 100 μmol/l MgCl₂.

Competition by several agonists and antagonists for [3H]CCPA binding was measured to confirm that [3H]CCPA binds to an A₁ adenosine receptor. The Kᵦ-values exhibit the typical pharmacological profile for A₁ receptors with the marked stereoselectivity for the PIA enantiomers and high affinity binding of DPCPX (Table 1).

The replacement of the 5'-hydroxyl by a 5'-chloro substituent at N⁶-substituted adenosine derivatives has been reported to enhance A₁ receptor selectivity (Taylor et al. 1986). This additional modification, which leads to CCCPA, did not further increase A₁ affinity compared to CCPA (Table 1). Binding data for CCCPA at A₂ receptors were also very similar to the data for CCPA (not shown). A Kᵦ-value of 0.29 nmol/l was determined for [3H]CCCPA at A₁ receptors of rat brain membranes (Table 2).

The time course of association of [3H]CCPA to solubilized A₁ receptors from rat brain membranes was mea-
Table 2. Comparison of agonist radioligands for A₁ adenosine receptors of rat brain membranes

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>$K_D$ (nmol/l)</th>
<th>Specific activity (Ci/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]PIA</td>
<td>1.4*</td>
<td>49</td>
</tr>
<tr>
<td>[³H]CCPA</td>
<td>0.5*</td>
<td>46</td>
</tr>
<tr>
<td>[³H]CCCPA</td>
<td>0.3</td>
<td>43</td>
</tr>
<tr>
<td>[³H]CCPA</td>
<td>0.2</td>
<td>43</td>
</tr>
</tbody>
</table>

* Data from Lohse et al. (1984)
<table>
<thead>
<tr>
<th>Radioligand</th>
<th>$K_D$ (nmol/l)</th>
<th>Specific activity (Ci/mmol)</th>
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<td>[³H]PIA</td>
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<td>[³H]CCCPA</td>
<td>0.3</td>
<td>43</td>
</tr>
<tr>
<td>[³H]CCPA</td>
<td>0.2</td>
<td>43</td>
</tr>
</tbody>
</table>

* Data from Williams et al. (1986)

Table 3. $K_D$-values for [³H]CCPA at A₁ receptors from different rat tissues. The data are geometric means with 95% confidence limits from 3 – 4 separate experiments

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$K_D$ (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain membranes</td>
<td>0.21 (0.19 – 0.23)</td>
</tr>
<tr>
<td>Solubilized receptors (control)</td>
<td>0.24 (0.14 – 0.39)</td>
</tr>
<tr>
<td>Solubilized receptors (MgCl₂)</td>
<td>0.15 (0.05 – 0.42)</td>
</tr>
<tr>
<td>Myocyte membranes</td>
<td>0.43 (0.21 – 0.88)</td>
</tr>
</tbody>
</table>

Binding of [³H]CCPA was tested in human platelets to examine whether this radioligand retained the high A₁ selectivity of the nonradioactive compound. Both with platelet membranes and solubilized A₁ receptors no specific binding of [³H]CCPA was observed at concentrations up to 400 nmol/l (not shown).

Discussion

Different modifications at the N⁶-position of adenosine led in the past to agonists with high affinity and selectivity for A₁ adenosine receptors. In particular, the N⁶-cyclopentyl analogue of adenosine, CPA, is a potent and A₁-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 A₁ selectivity (Cristalli et al. 1988). This observation led subsequently to the synthesis of CCPA with an almost 10000-fold selectivity for the A₁ 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. [³H]CCPA exhibits subnanomolar affinity for A₁ 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 [³H]CCPA is GTP sensitive in a manner characteristic for agonists at G protein-coupled receptors. Competition of several agonists and antagonists for [³H]CCPA binding showed the pharmacological profile for an A₁ adenosine receptor. In particular the stereoselectivity for the PIA enantiomers and the high affinity binding of DPCPX demonstrated that [³H]CCPA labels A₁ receptors. It has been shown that the association rate of [³H]PIA at solubilized A₁ receptors is markedly attenuated by Mg²⁺-ions (Klotz et al. 1986). This was also observed for [³H]CCPA suggesting that this radioligand possesses all the characteristics of an A₁ receptor agonist.

Trivedi et al. (1989) described recently [³H]S-ENBA ([³H]R,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 [³H]CCPA has first of all a higher specific radioactivity and exhibits in addition a slightly higher affinity at A₁ receptors.

In analogy to the 2-chloro modification of CPA a 5'-chloro-5'-deoxy modification of N⁶-substituted adenosine derivatives was reported to also increase A₁ selectivity (Taylor et al. 1986; Trivedi et al. 1989). We therefore synthesized CCCCAPA as a derivative with both modifications. No additional increase in A₁ selectivity or A₁ affinity occurred. Likewise, [³H]CCCPA was not superior to [³H]CCPA.

Detection of A₁ receptors in tissues with very low receptor density has been successful so far only with [³H]DPCPX (Lohse et al. 1987) or with radioiodinated agonists (Linden et al. 1985; Lohse et al. 1985). The high affinity of [³H]CCPA suggested that this radioligand might be advantageous for labelling of A₁ receptors in tissues like rat heart, where only 18 fmol/mg protein have been found with [³H]PIA (Martens et al. 1987). Saturation experiments proved that [³H]CCPA is an agonist radioligand which can be used instead of radioiodinated agonists for the detection of A₁ receptors in tissues with low receptor density.

No binding of [³H]CCPA, at concentrations up to 400 nmol/l, was observed to both membrane-bound or...
solubilized A₂ receptors from human platelets. It can be roughly estimated that the Kᵦₜ-value of [³H]CCPA at A₂ receptors should be higher than 4 μmol/l. Thus, the about 10000-fold A₁-selectivity of CCPA seemed to be preserved for the tritiated compound.

In summary, it is concluded that [³H]CCPA is a new agonist radioligand with high selectivity for A₁ receptors, exhibiting virtually no affinity for A₂ receptors. The sub-nanomolar affinity for A₁ receptors makes [³H]CCPA an important tool for the characterization of receptors in different tissues, in particular for tissues with very low receptor density.

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