[3H]-8-Cyclopentyl-1,3-dipropylxanthine
Binding to A\textsubscript{1} Adenosine Receptors of Intact
Rat Ventricular Myocytes

Dirk Martens, Martin J. Lohse, and Ulrich Schwabe

The purpose of the present study was the identification of A\textsubscript{1} adenosine receptors in intact rat ventricular myocytes, which are thought to mediate the negative inotropic effects of adenosine. The adenosine receptor antagonist [3H]-8-cyclopentyl-1,3-dipropylxanthine was used as radioligand. Binding of the radioligand to intact myocytes was rapid, reversible, and saturable with a binding capacity of 40,000 binding sites per cell. The dissociation constant of the radioligand was 0.48 nM. The adenosine receptor antagonists 8-cyclopentyl-1,3-dipropylxanthine, "xanthine amine congener," and theophylline were competitive inhibitors with affinities in agreement with results obtained for A\textsubscript{1} receptors in other tissues. Competition experiments using the adenosine receptor agonists R-N\textsuperscript{6}-phenylisopropyladenosine, 5'-N-ethylcarboxamidoadenosine, and S-N\textsuperscript{2}-phenylisopropyladenosine gave monophasic displacement curves with K\textsubscript{d} values of 50 nM, 440 nM, and 4,300 nM, which agreed well with the GTP-inducible low affinity state in cardiac membranes. The low affinity for agonists was not due to agonist-induced desensitization, and correlated well with the corresponding IC\textsubscript{50} values for the inhibition of cyclic AMP accumulation by isoprenaline. It is suggested that only a low affinity state of A\textsubscript{1} receptors can be detected in intact rat myocytes due to the presence of high concentrations of guanine nucleotides in intact cells. (Circulation Research 1988;63:613–620)

Membrane-bound adenosine receptors, which mediate coronary dilation, negative chronotropic, dromotropic, and inotropic effects have been demonstrated in the heart (see Daly\textsuperscript{1} for a review). Depending on their coupling to adenylate cyclase, two subtypes of adenosine receptors can be distinguished. The A\textsubscript{1} receptor mediates an inhibition of adenylate cyclase, whereas the A\textsubscript{2} subtype mediates an activation.\textsuperscript{2} In cardiac myocytes, adenosine receptors negatively coupled to adenylate cyclase, and therefore belonging to the A\textsubscript{1} subtype, have been demonstrated,\textsuperscript{3} whereas A\textsubscript{2} adenosine receptors have been demonstrated on the coronary endothelium\textsuperscript{4} and coronary smooth muscle.\textsuperscript{5}

Subsequently, cardiac A\textsubscript{1} receptors have further been characterized by radioligand binding to myocardial membranes through the use of the adenosine receptor agonists R-N\textsuperscript{6}-[\textsuperscript{125}I]p-hydroxyphenylisopropyladenosine and [\textsuperscript{125}I]N\textsubscript{2}-4-aminobenzyladenosine.\textsuperscript{6,7}

Recently, the A\textsubscript{1}-selective antagonist radioligand [3H]-8-cyclopentyl-1,3-dipropylxanthine ([3H]DPCPX) has been shown to be a suitable radioligand for the characterization of A\textsubscript{1} adenosine receptors of bovine myocardial membranes.\textsuperscript{8}

However, the results obtained in membrane preparations may not reflect physiological conditions, and cell disruption could induce changes in receptor properties and regulation. Therefore, we attempted to identify adenosine receptors in intact rat ventricular myocytes by measurement of [3H]DPCPX binding.

Materials and Methods

[3H]DPCPX (specific activity 105 Ci/mmol) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were synthesized as described earlier\textsuperscript{8} and were diluted in 0.1% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate to reduce adsorption to surfaces. This addition did not affect the respective assays. R- and S-N\textsuperscript{6}-phenylisopropyladenosine (R-PIA and S-PIA) were donated by Dr. Stegmeier, Boehringer Mannheim GmbH, Mannheim, FRG; 5'-N-ethylcarboxamidoadenosine (NECA) was provided by Prof. Klemm, Byk Gulden Lomberg Chemische Fabrik, Konstanz, FRG; and 8-{4-[{{(2-
aminoethyl)amino]carbonyl[methyl]oxyl)phenyl]-1,3-dipropylxanthine (xanthine amine congener, XAC) was a kind gift from Dr. Entzeroth, Dr. Karl Thoma GmbH, Biberach, FRG. Theophylline, 3-(p-nitrobenzyl)-6-thioinosine (NBTI), bovine serum albumin fraction V, and crude collagenase type 1 were from Sigma, Deisenhofen, FRG. All other materials were from sources described previously and of analytical grade or best commercially available.

Isolation of Rat Ventricular Myocytes

Calcium-tolerant myocytes were isolated as previously described. Adult male Wistar rats (120-250 g) were injected with 500 units sodium heparin i.p. followed by 60 mg/kg sodium pentobarbital i.p. The hearts were rapidly excised, mounted on a cannula of a Langendorff perfusion system, and perfused for 5 minutes at a pressure of 50 cm water with a calcium-free Krebs-Ringer bicarbonate-glucose buffer (KRBG-buffer) containing (mM) NaCl 118, KCl 4.74, KH2PO4 0.93, MgSO4 1.2, NaHCO3 25, and glucose 10 equilibrated at 37°C with 95% O2-5% CO2 and adjusted to pH 7.4. All subsequent steps were done at 37°C. The hearts were perfused for 10 minutes in a recirculating manner with 50 ml KRBG-buffer containing 0.1% bovine serum albumin and 0.05% collagenase. The ventricles were then cut down, two vertical slashes were made toward the apex, and the tissue was incubated in KRBG-buffer supplemented with 2% bovine serum albumin and 0.1% collagenase under gentle shaking. After 5 minutes, the undigested tissue was again incubated in a new portion of the same solution for 10 minutes as before. The resulting suspension was then filtered through a 200-μm nylon gauze and centrifuged (25g, 90 seconds), and the sediment was washed twice with KRBG-buffer containing 2% bovine serum albumin. Finally, the pelleted tissue was resuspended in 50 mM Tris-HCl buffer (4°C). The samples were kept on ice for 3 minutes, followed by rapid filtration through Whatman GF/B glass fiber filters and four washes with 4-ml portions of ice-cold incubation buffer. The filter radioactivity was determined by liquid scintillation counting for 10 minutes. Non-specific binding was defined by the presence of 1 mM theophylline. The addition of 4 ml buffer and storage on ice before filtration resulted in a reduction of non-specific binding by more than 70% with only a slight (10%) lowering of specific binding. In typical experiments in which a radioligand concentration of 0.2 nM [3H]DPCPX was used, total binding was approximately 550 cpm compared with 120 cpm non-specific binding. All assays were done in duplicate.

Binding of [3H]DPCPX to myocardial membranes was done in a volume of 250 μl of 50 mM Tris-HCl (pH 7.4) with the same additions described above and approximately 200 μg of membrane protein. Prior to the start of the incubation, myocardial membranes were suspended in 50 mM Tris-HCl and 10 mM EDTA (pH 7.4) to remove endogenous divalent cations, centrifuged (100,000g, 10 minutes, 4°C), and the pellet resuspended in 50 mM Tris-HCl (pH 7.4). Incubation was carried out at 25°C for 1 hour. The end of the incubation, a 200-μl aliquot was filtered through a Whatman GF/B glass fiber filter, followed by two washes with 4-ml portions of 50 mM Tris-HCl (pH 7.4, 4°C). Non-specific binding was defined as above, and at a radioligand concentration of 0.2 nM [3H]DPCPX was approximately 60 cpm compared with 300 cpm of total binding.

Measurement of Cyclic AMP

The cyclic AMP content of ventricular myocytes was measured as previously described. Briefly, rat ventricular myocytes (approximately 15,000 cells/tube) were incubated in a total volume of 1 ml KRBG-buffer supplemented with 10 mM HEPES and 1.4 mM CaCl2. Further additions were 0.5 units/ml adenosine deaminase, 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), and 1 μM isoprenaline. For the calculation of Kd values, dose-response curves of R-PIA in the presence of three different concentrations of the respective adenosine receptor antagonist were performed. The incubation lasted 10 minutes at 37°C and was terminated by the addition of 200 μl of 3.9 M HClO4. Cyclic AMP was determined by a sensitive radioimmunoassay.

Data Analysis

Radioligand binding data were analyzed with the nonlinear curve-fitting program SCTFTT. Kinetic data were fitted as previously described. Kinetics in the presence of a competitor were fitted using Equation 1 of Matulsky and Mahan, using the general exponential equation previously given. Concentration response curves were fitted to the
Hill equation by a nonweighted nonlinear curve fitting program as described earlier. The apparent dissociation constant of competitors $K_d$ was calculated according to Arunlakshana and Schild.

**Results**

**Binding of [3H]DPCPX to Rat Ventricular Myocytes**

The specific binding of 0.2 nM [3H]DPCPX to rat ventricular myocytes occurred rapidly with half-maximal binding after 3 minutes and achievement of equilibrium after 30 minutes (Figure 1). Nonspecific binding in the presence of 1 mM theophylline reached a steady state after 2 minutes and contributed approximately 20% to total binding at equilibrium (data not shown). Nonlinear curve-fitting of the association reaction of specific binding gave no improvement of the fit when two components were assumed. Therefore, the association of the radioligand appears to be monophasic, indicating a homogeneity of binding sites. The observed forward rate constant ($k_+$) was calculated to 0.165/min, giving an association constant ($k_+$) of 0.233/nM/min.

The radioligand binding was reversible as demonstrated by the addition of an excess of theophylline at equilibrium (Figure 1). The dissociation occurred with a half-life of 6 minutes, corresponding to a dissociation rate constant ($k_-$) of 0.124/min. The ratio $k_+ / k_- = 0.53$ nM and provides an estimate of the equilibrium dissociation constant $K_d$, independent from saturation experiments.

Data from saturation experiments are shown in Figure 2. The specific binding of [3H]DPCPX was saturable. The Scatchard plot of the data is linear, indicating again a homogeneous population of noncooperative binding sites with a binding capacity ($B_{max}$ value) of 68 fmol/10^6 cells. Assuming that one molecule of [3H]DPCPX binds to one receptor, the total number of receptors was found to be 40,000/cell. The $K_d$ value was calculated to 0.48 nM, which is in excellent agreement with the $K_d$ value calculated from kinetic experiments. It can be seen that nonspecific binding constitutes only 35% of total binding at the $K_d$.

Further characterization of [3H]DPCPX binding sites on rat ventricular myocytes was achieved by...
competition experiments using the adenosine receptor agonists R-PIA, NECA, and S-PIA (Figure 3 and Table 1). The competition curves were monophasic with slope factors not different from unity. The A1 receptor-selective compound R-PIA was the most potent agonist with a $K_i$ value of 50 nM, whereas NECA had a ninefold lower affinity, which is characteristic for A1 adenosine receptors, as opposed to A2 receptors. The S-isomer of PIA was about 100 times less potent than the R-isomer in displacing $[^3H]$DPCPX from its binding sites, indicating a high stereospecificity.

A high affinity state for R-PIA with a $K_i$ value below 1 nM, as described in bovine myocardial membranes, could not be detected. Since competition experiments were performed under equilibrium conditions (1 hour of incubation), the lack of a high affinity state for agonists might be due to an agonist-induced desensitization. This phenomenon has been described for muscarinic and $\beta$-adrenergic receptors.16,17 To determine whether incubation of intact myocytes with an agonist induces a conversion of A1 receptors from a high to low affinity state the experimental procedure previously described by Nathanson was used.18 Kinetics of the binding of $[^3H]$DPCPX to intact myocytes were measured in the absence or presence of 10 nM R-PIA (Figure 4). This concentration caused only a 6% reduction of specific binding at equilibrium but up to 25% reduction during the first 5 minutes of incubation. If this transient larger inhibition of $[^3H]$DPCPX binding were caused by the presence of a high agonist affinity form, then preincubation with 10 nM R-PIA should cause conversion to the low affinity state before the addition of the radioligand, therefore resulting in a smaller initial inhibition of $[^3H]$DPCPX

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<tr>
<td>GTP</td>
<td>&gt;100,000 (-6%)</td>
</tr>
<tr>
<td>Inosine</td>
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*Data from Martens et al.*

$K_i$ values were calculated from competition experiments using 8-11 concentrations of the displacing compound. Estimates of the high ($K_{H}$) and low ($K_{L}$) affinity dissociation constants and the percentage of total receptors in a high affinity state ($R_H$) are given, if a two-site model gave a significantly better fit. $K_{H}$, apparent dissociation constant, calculated from Schild plot.
binding. Preincubation with R-PIA did not alter the transient inhibition of $[^3H]$DPCPX binding. This suggests that the initial inhibition is a result of a higher association rate of 10 nM R-PIA than of 0.2 nM $[^3H]$DPCPX. However, these experiments do not rule out the possibility that agonist-induced conversion from a high to a low agonist affinity state occurs in a time scale too rapid to be detectable in these experiments.

Several alkylxanthines competed for $[^3H]$DPCPX binding to myocytes with slope factors of about 1. DPCPX was the most potent antagonist, being about 100 times more potent than XAC and 10,000 times more potent than theophylline (Table 1).

In order to exclude that $[^3H]$DPCPX binds to adenosine uptake sites, or that $[^3H]$DPCPX binding to intact cells is mainly due to intracellular uptake facilitated by the adenosine transport system, we investigated the influence of the adenosine uptake blocker NBTI on $[^3H]$DPCPX binding to rat ventricular myocytes. Even a high concentration of NBTI (10 $^\mu$M) caused only a 12% reduction of radioligand binding. In addition, inosine, a degradation product of adenosine, and GTP failed to modulate $[^3H]$DPCPX binding to rat ventricular myocytes.

**Binding of $[^3H]$DPCPX to Rat Myocardial Membranes**

$[^3H]$DPCPX binding sites were further characterized in myocardial membranes in order to determine differences between binding to membranes and whole cells. All antagonists exhibited monophasic displacement curves with slope factors of about 1, indicating one homogeneous class of binding sites. The $K_i$ values (Table 1) were not significantly different from those obtained in experiments using intact myocytes, with DPCPX being more potent than XAC and theophylline.

However, the competition curves for agonists, as demonstrated in Figure 5 for R-PIA, were shallow and could be fitted significantly better to a two-site model ($p<0.001$, $F$ test), which indicates that agonists can discriminate two affinity states of $[^3H]$DPCPX binding sites in myocardial mem-
branes. Identical results were obtained if membrane preparations from rat ventricular myocytes instead of myocardial membranes were used. Therefore, these results were not due to a noncardiomyocyte contamination. The \( K_i \) values of the low affinity site for agonists (\( K_i \)) were about 50 to 80 times higher than the \( K_i \) values for the high affinity site (\( K_{iH} \)) (Table 1). There was good agreement of the \( K_i \) values of membranes and the \( K_i \) values obtained from \([3H]\)DPCPX binding to intact myocytes.

The use of KRBG-buffer instead of 50 mM Tris- HCl (pH 7.4) for \([3H]\)DPCPX binding to cardiac membranes altered neither the \( K_i \) values nor the amount of receptors in the high and low affinity states. Therefore, it is concluded that the absence of a high agonist affinity state is not the result of different buffer systems used with intact cells.

Under standard assay conditions about two thirds of the \([3H]\)DPCPX binding sites in rat myocardial membranes were in a high affinity state for agonists, and GTP caused a shift toward the low affinity state (Figure 5). In the presence of 100 \( \mu \)M GTP, only 14% of the \([3H]\)DPCPX binding sites remained in the high affinity state for the agonist \( R \)-PIA.

**Effect of Various Adenosine Receptor Agonists and Antagonists on Isoprenaline-Mediated Cyclic AMP Accumulation**

To compare the results from \([3H]\)DPCPX binding to rat ventricular myocytes and cardiac membranes with functional parameters, we have examined the effect of various adenosine agonists and antagonists on isoprenaline-mediated cyclic AMP accumulation. As shown in Figure 6, \( R \)-PIA caused a reduction of cyclic AMP content of myocytes by up to 50% with an \( IC_{50} \) value of 60 nM. Using different concentrations of DPCPX a dose-dependent shift of the concentration-response curve of \( R \)-PIA to higher concentrations was observed without alteration of the slope and the maximal effect of \( R \)-PIA. The apparent dissociation constant (\( K_B \)) of DPCPX was calculated to 0.15 nM, which is in good agreement with the corresponding data from radioligand binding.

In addition, the \( IC_{50} \) and \( K_B \) values of various adenosine agonists and antagonists for the modulation of cyclic AMP content of rat ventricular myocytes via \( A_1 \) receptors (Table 1) were in fairly good agreement with their \( K_i \) values obtained in \([3H]\)DPCPX binding to intact myocytes.

**Discussion**

Binding of \([3H]\)DPCPX to \( A_1 \) adenosine receptors in membrane preparations has been described in several tissues including brain and heart. This radioligand has a more than 700-fold selectivity for the \( A_1 \) subtype, a very low nonspecific binding, and a high specific activity. This led us to attempt a characterization of \( A_1 \) receptors in intact cells with this radioligand.

The present study demonstrates \([3H]\)DPCPX binding sites on rat ventricular myocytes, which possess properties characteristic for \( A_1 \) adenosine receptors. The binding of \([3H]\)DPCPX to its binding sites was rapid, reversible, and saturation with a binding capacity of 40,000 binding sites per cell. Therefore, the receptor density is lower than that reported for muscarinic, insulin, and \( \beta \)-adrenergic receptors on myocytes, which are at least twofold higher.

Evidence that \([3H]\)DPCPX binds to \( A_1 \) receptors in intact myocytes was established in competition experiments with the adenosine receptor antagonists DPCPX, XAC, and theophylline. All alkylxanthines had only one class of binding sites with identical affinities in cardiac membranes and myocytes. In addition, the \( K_i \) values obtained are in good agreement with those reported in other tissues. Furthermore, these compounds antagonize the \( A_1 \) receptor–mediated reduction of cyclic AMP levels with the same affinities. The usefulness of alkylxanthines for the characterization of adenosine recep-
tors is demonstrated by the independence of the $K_i$ values from the method used.

The potencies of the adenosine receptor agonists in competing for the binding sites of myocytes were in the order $R$-PIA > NECA > S-PIA, consonant with an interaction at $A_1$ receptors. The same rank order of potency has been observed for the inhibition of the isoprenaline-mediated cAMP-accumulation in rat ventricular myocytes. In addition, the affinities of the adenosine agonists in intact myocytes agree well with the low affinity state in membranes.

In addition to a single class of low agonist affinity [$^3H$]DPCPX binding sites in intact rat ventricular myocytes, we have recently shown by [$^3H$]R-N$^6$-phenylisopropyladenosine binding to $A_1$ receptors of intact rat adipocytes, that there was no detectable high affinity binding for agonists in intact cells, either. Therefore, this seems to be a general phenomenon of $A_1$ adenosine receptors.

There is a good correlation between the $K_i$ values of agonists in intact cells and the $IC_{50}$ values for the reduction of cyclic AMP accumulation. This suggests that the functional response is initiated by binding of the agonists to a low affinity state. The virtual absence of the high agonist affinity state indicates that the interaction with $G_i$ is a very transitory process, followed by rapid conversion to the low affinity state, presumably by binding of GTP, causing dissociation of $G_i$ and the receptor. Similar findings were recently reported for the $A_1$ adenosine receptor in isolated rat adipocytes.

In agreement with our results it is also true in the case of muscarinic receptors that only low affinity binding sites can be detected in intact cardiac myocytes, whereas in myocyte membranes also a high affinity state was detectable. The low affinity form could be induced by GTP and was not due to desensitization. It was concluded that the low affinity state is the physiologically active form of muscarinic receptors.

The affinities of $R$-PIA and NECA for [$^3H$]DPCPX binding sites on intact rat ventricular myocytes correlate well with their potencies to inhibit cyclic AMP accumulation, and are also in the same range as the $IC_{50}$ values of their negative inotropic effects. This contrast with the $A_1$ receptor system of adipocytes: here the [$^3H$]DPCPX values of agonists for the reduction of cyclic AMP levels are 20-fold lower than the corresponding $K_i$ values determined in binding experiments. From that and additional evidence, it was concluded, that adipocytes have spare $A_1$ receptors. In contrast, it may be reasoned from the same argument that the good correlation between the affinity of agonists for $A_1$ receptors of intact myocytes and their $IC_{50}$ values for the reduction of cyclic AMP levels indicates the absence of spare $A_1$ receptors in ventricular myocytes.

In summary, our results indicate, that [$^3H$]DPCPX is the first radioligand suitable for the characterization of $A_1$ receptors on intact myocytes. $A_1$ receptors on intact cells seem to be in a low affinity state for agonists.

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**References**


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**Radloligand Binding to Cardiac $A_1$ Receptors 619**


**KEY WORDS** • adenosine receptors • ventricular myocytes • radioligand binding • xanthines
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