Electrophysiological and Receptor Binding Studies to Assess Activation of the Cardiac Adenosine Receptor by Adenine Nucleotides

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Adenosine and adenosine nucleotides shorten the action potential duration of atrial myocytes and activate a specific acetylcholine and adenosine receptor–operated potassium outward current referred to as I_{K_{ACH,Ado}}. The objective of this study was to determine whether adenine nucleotides shorten the action potential duration and increase I_{K_{ACH,Ado}} in guinea pig atrial myocytes by directly activating adenosine receptors. The potency and efficacy of AMP and adenosine in increasing I_{K_{ACH,Ado}} and shortening atrial action potential duration were similar; the EC_{50} values for AMP and adenosine were 3.4±0.8 and 3.1±0.4 μM, respectively. Likewise, the maximum increases in I_{K_{ACH,Ado}} caused by AMP and adenosine were similar (122±11% versus 123±9%). In comparison, ATP and the stable analogue of AMP, adenosine monophosphorothioate (AMPS), were significantly less potent and efficacious than adenosine and AMP, and inosine had no effect. The electrophysiological effects of AMP were antagonized by the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline and abolished in the presence of adenosine deaminase and α,β-methylene-ADP (APCP, an inhibitor of AMP degradation). Binding of the A_{1}-adenosine antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine (DPCPX) to guinea pig atrial membranes treated with adenosine deaminase and APCP was reduced up to 60% by 100 μM concentrations of AMP, AMPS, and adenosine. Inosine inhibited binding by 43±3% at 100 μM, whereas hypoxanthine and xanthine had little (5–10% inhibition) and uric acid had no effect. Only 3% of AMP and 35% of AMPS had been recovered intact after a 90-minute incubation at 21°C with preparations of guinea pig atrial membranes. Percent displacement of [^{3}H]DPCPX binding to atrial membranes by 100 μM AMP was significantly less in the presence of nucleoside phosphorylase and xanthine oxidase (to degrade inosine, hypoxanthine, and xanthine to uric acid) than in their absence (12.4±3.1% versus 49.7±1.5%). The results suggest that the observed electrophysiological actions of adenine nucleotides in cardiomyocytes are mediated by adenosine and are consistent with activation of A_{1}-adenosine receptors. (Circulation Research 1991;68:1035–1044)

Adenosine has been shown to slow heart rate and atrioventricular conduction via activation of an outward K^{+} current or by antagonism of catecholamine-stimulated inward calcium current and pacemaker current.1–4 These actions are initiated on activation of specific A_{1}-adenosine receptors on the surface of the cell.

Adenine nucleotides are released from myocardial and endothelial cells during pharmacological stimulation and pathophysiological situations,5–7 and some of their cardiac effects are similar to those of adenosine.8,9 Enzymes that catalyze the degradation of adenine nucleotides are present on the extracellular membrane surfaces of cardiac, smooth muscle, and vascular cells.10–15 Therefore, extracellular ATP, ADP, and AMP are rapidly and sequentially degraded to adenosine.

Previous studies,9,16 including those of the authors,8 have indicated that under specific experimental conditions the effects of adenine nucleotides depend on their degradation to adenosine. Thus, the effects of adenine nucleotides on atrial myocyte membrane potential and ionic currents may involve activation of adenosine receptors by the nucleotides per se or activation of adenosine receptors by adenosine formed on degradation of these nucleotides (Figure 1). To determine which of these occurs, electrophysiological studies on isolated guinea pig...
atrial myocytes and radioligand binding competition experiments for A1-adenosine receptors in bovine brain and guinea pig cardiac tissues were performed.

Materials and Methods

Adenosine, AMP, ATP, α,β-methylene-ADP (APCP), adenosine monophosphorothioate (AMPS), N^6-cyclopentyladenosine (CPA), (−)-N^6-(2-phenylisopropyl)adenosine (R-PIA), adenosine deaminase (ADA) type VI, and other nucleotides and nucleosides were purchased from Sigma, St. Louis. 8-(p-Sulfonylphenyl)theophylline (8-PST) was purchased from Research Biochemicals, Natick, Mass. [³H]8-Cyclopentyl-1,3-dipropylxanthine ([³H]DPCPX; specific activity, 99–107 Ci/μmol) was purchased from Amersham, Arlington Heights, Ill. Rabbit antiserum against 5’-nucleotidase was a kind gift of Dr. Andrew Newby, Department of Cardiology, Welsh National School of Medicine, Heath Park, Cardiff, Wales.

Isolated Atrial Myocytes

Guinea pig atrial myocytes were isolated by enzymatic dissociation as previously described.³ Briefly, hearts were quickly removed, rinsed, mounted in a Langendorff apparatus, and perfused with modified Krebs-Henseleit (K-H) solution containing (mM) NaCl 127, KCl 4.6, CaCl₂ 2, MgSO₄ 1.1, sodium pyruvate 2, glucose 10, creatine 10, taurine 20, ribose 5, adenine 0.01, HEPES 5, and allopurinol 0.1, pH 7.4. Hearts were enzymatically digested at 35°C during perfusion for 5–10 minutes with oxygenated Ca²⁺-free K-H solution containing (mg/ml) dispase 0.1, trypsin 0.12, collagenase 0.32, and albumin 2.12. After removal of the heart from the cannula, the atria were dissected out, minced, and incubated at 37°C with fresh enzyme solution in a shaker bath. Suspensions containing intact myocytes were pooled, and the myocytes were allowed to settle. Enzyme solution was removed, and cells were resuspended and stored in K-H solution containing 100 μM Ca²⁺ at room temperature.

Electrophysiological Measurements

Isolated quiescent, relaxed cells with clear striations and rod shape when exposed to K-H solution with 2 mM calcium were used for experimentation. The enzymatically dispersed atrial myocytes were transferred into a recording chamber, allowed to settle to the bottom of the chamber, and superfused with K-H solution at a flow rate of 2–4 ml/min (35±1°C), as previously described.³,¹⁷ Both membrane potentials and ionic currents were recorded with glass suction pipettes, that is, “patch electrodes” (Kimax glass pipettes, Kimble Glass, Vineland, N.J.), in a whole-cell configuration as described by Hamill et al.¹⁸ The recording electrodes had resistances of 2–4 MΩ when filled with the following internal pipette solution (mM): KCl 10, potassium aspartate 130, Na₂ATP 4, MgCl₂ 1, HEPES 10, and sodium EGTA 1, pH 7.2. Junction potentials between the internal pipette solution and extracellular medium were nulled before seal formation.

Data Recording and Analysis of Electrophysiological Experiments

Recordings were made with an Axopatch-1C amplifier (Axon Instruments, Burlingame, Calif.). Action potentials and membrane currents were displayed on a storage oscilloscope (model 5113, Tektronix, Beaverton, Ore.). Subsequently the signals were digitized using a 2801-A board (Data Translation Inc., Marlboro, Mass.) and stored on a microcomputer hard disk.

Action potential durations (in milliseconds) were measured at the 0 mV level (APD₀) and at 90% of repolarization (APD₉₀). Late outward current, an absolute value of the current with respect to a base line of 0 nA at the end of a 150-msec clamp pulse, was attributed to potassium. The acetycholine- or adenosine-induced increase in the potassium current in atrial myocytes is the result of activation of a specific outward potassium current referred to herein as Iₖᵦᵦ,₂⁺.¹⁹

Membrane Preparation

Brain. Pieces of frozen bovine brain cortex (Pel-Freeze Biologicals, Rogers, Ark.) were immediately immersed and homogenized (Polytron, Brinkmann Instruments, Westbury, N.Y.) for 10 seconds in 10 vol ice-cold buffer, pH 7.4, containing 10 mM HEPES, 10 mM EDTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 10 μg/ml phenylmethylsulfonyl fluoride, and 10% (wt/vol) sucrose. The homogenate was filtered through 210-μm nylon mesh and centrifuged at 30,000g for 15 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 vol of the same buffer and washed three times by centrifugation. The washed pellet was resuspended to give a protein concentration of 0.4–0.6 mg/ml in 25
mM Bis-Tris propane buffer, pH 7.4, containing 1 mM MgCl₂.

Heart. Guinea pig hearts were perfused for 10 minutes with oxygenated K-H solution to remove blood. After a rinse in ice-cold HEPES buffer containing 10 μg/ml leupeptin, atria or ventricles were isolated and homogenized (Polytron, Brinkmann Instruments) for 10 seconds in 10 vol of the same buffer, filtered through four layers of gauze, and centrifuged at 30,000g for 15 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 100 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂, to give a protein concentration of 2–3 mg/ml.

Binding Assay

Membranes were incubated with ADA (2 units/ml = 12.5 μg/ml) for 30 minutes at 22°C. ADA was not added in some experiments in which membranes were incubated with adenosine. A₁-Adenosine binding sites were measured using [³H]DPCPX as radiolabeled ligand. Membranes (50–150 μl) and labeled ligand with or without unlabeled competing ligands were incubated in a total volume of 250 μl. Incubations were carried out at 21°C for 90 minutes on an orbital shaker. Radioligand concentrations were 1–2 nM for brain membranes and 4 nM for cardiac membranes. Incubations were terminated by rapid collection (Brandel Cell Harvester M-24R, Brandel Scientific, Gaithersburg, Md.) of membranes on GF/C glass fiber filters (Whatman Inc., Clifton, N.J.) presoaked with 0.1% CHAPS to reduce nonspecific binding. The filters were washed three times in 10 seconds with 4-ml aliquots of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and counted in 10 ml scintillation cocktail (Scintiverse II, Fisher Scientific Co., Pittsburgh, Pa.). Nonspecific binding was defined as the amount of radioligand bound in the presence of 10 μM R-PIA.

Adenine Nucleotide Stability Experiments

To evaluate the stability of adenine nucleotides added to membrane preparations, experiments were conducted to determine recovery of added nucleotides. Membranes from bovine brain (protein content, 0.4–0.6 mg/ml) and from guinea pig ventricles or atria (protein content, 2–2.5 mg/ml) were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 2 units/ml ADA. AMP or AMPS plus APCP were added to membrane preparations and incubated at 21°C for 90 minutes. After incubation, samples were centrifuged at 11,000g for 1 minute. A 900-μl aliquot of supernatant was removed, added to 100 μl of 5 M HClO₄ in a microtube, and centrifuged at 11,000g for 10 minutes. The supernatant (900 μl) was added to 105 μl of 5 M KOH in a microtube, then centrifuged at 11,000g for 10 minutes, and titrated to pH 7–7.5. A 100-μl aliquot was injected onto a reversed-phase Ultrasphere ODS 4.5×250-mm column (Beckman Instrument...
Adenosine nucleotides on membrane potential and action potential duration were similar to those of adenosine. As shown in the upper panels of Figure 3, AMP and ATP shortened the duration of the atrial action potential. AMP (10 \mu M) decreased the APD\(_{90}\) from a control of 86 msec to 24 msec (Figure 3A), whereas ATP (100 \mu M) caused a smaller decrease in APD\(_{90}\) from a control of 90 msec to 55 msec (Figure 3C). Likewise, AMP caused a greater increase in I\(_{\text{KACH,Ado}}\) than did ATP. For example, 10 \mu M AMP increased I\(_{\text{KACH,Ado}}\) by 1.0 nA, that is, from 1.5 to 2.5 nA (Figure 3B). In comparison, 100 \mu M ATP increased I\(_{\text{KACH,Ado}}\) by only 0.4 nA (Figure 3D). Inosine (30 \mu M), a metabolite of adenosine (Figure 1), had a negligible electrophysiological effect (Figure 2).

Concentration–response curves for adenosine, AMP, and ATP effects on I\(_{\text{KACH,Ado}}\) and APD\(_{90}\) are shown in Figure 4. The concentrations of adenosine and AMP to increase I\(_{\text{KACH,Ado}}\) by 50% were almost identical (i.e., 3.1±0.4 versus 3.4±0.8 \mu M). The maximal increases in I\(_{\text{KACH,Ado}}\) caused by adenosine and AMP did not differ (123±9% versus 122±11%). In contrast, ATP had a significantly lower potency and efficacy in increasing I\(_{\text{KACH,Ado}}\) compared with adenosine and AMP (Figure 4A). The effects of adenosine, AMP, and ATP on APD\(_{90}\) (Figure 4B) were similar to those on I\(_{\text{KACH,Ado}}\) (Figure 4A).

**Are the Electrophysiological Effects of AMP Dependent on Activation of the A\(_1\)-Adenosine Receptor?**

Because AMP and adenosine caused similar changes both in I\(_{\text{KACH,Ado}}\) and in the duration of the atrial action potential, we examined whether the specific adenosine receptor antagonist 8-PST could antagonize the electrophysiological effects of AMP. As illustrated in Figure 5, the effect of 10 \mu M AMP on action potential duration of isolated atrial cells was completely antagonized by 10 \mu M 8-PST.

**Is Adenosine Responsible for Electrophysiological Effects Attributed to AMP?**

To test the hypothesis that a metabolite of AMP, rather than AMP itself, causes the electrophysio-
logical response observed, we exposed cells to AMP in the presence of APCP, a 5'-nucleotidase inhibitor\(^23\) that slows enzymatic degradation of AMP to adenosine, and ADA, which degrades adenosine to inosine (Figure 1). As illustrated in Figure 6B, when APCP (100 \(\mu\)M) and ADA (2 units/ml) were added to the medium superfusing the isolated cardiomyocytes, AMP (30 \(\mu\)M) did not significantly affect APD\(_{90}\) (1.7 ± 0.6% decrease versus control). In contrast, AMP alone (30 \(\mu\)M) produced a large and significant decrease in APD\(_{90}\) (28 ± 3% decrease versus control, from 76 ± 3 to 54 ± 3 msec, \(n=4, p<0.05\)). APCP (100 \(\mu\)M) and ADA (2 units/ml) did not affect action potential duration (75 ± 3.5 msec for APD\(_{90}\) versus 76 ± 3 msec for control).

In a separate series of experiments, the electrophysiological effects of AMPs, an analogue of AMP with increased stability to enzymatic degradation, were investigated. Both the efficacy and potency of AMPs to shorten APD\(_{90}\) were markedly less than those of adenosine and AMP, but similar to those of ATP (Figure 4B).

### Binding Studies

**Brain tissue.** To define the affinity of nucleosides and nucleotides for the A\(_1\)-adenosine receptor, a preliminary study was carried out using bovine brain membranes. The bovine brain has a high density of adenosine receptors and a negligible amount of nonspecific binding, compared with heart membranes. Figure 7A shows the effect of adenine nucleotides (100 \(\mu\)M) and adenosine (100 \(\mu\)M) on \(^{3}H\)DPCPX (2 nM) binding to bovine brain membranes in the presence and absence of ADA (2 units/ml) and of APCP (100 \(\mu\)M). The effect of ADA on \(^{3}H\)DPCPX binding in the absence of competing ligands was variable. In three experiments, specific binding of \(^{3}H\)DPCPX was reduced from 0% to 50% in the absence of ADA, compared with a control with ADA present (2 units/ml). Presumably this result reflects differences in endogenous adenosine formation in the preparations. In contrast, displacement of \(^{3}H\)DPCPX binding by added ligands or potential ligands was similar in magnitude in all three experiments. In the absence of ADA and APCP, it was possible to observe an inhibition of \(^{3}H\)DPCPX binding by adenosine and adenine nucleotides (Figure 7A, first row) with an apparent rank order: adenosine > AMP > AMPS > ADP > ATP. APCP (100 \(\mu\)M) (second row) markedly reduced the inhibition of \(^{3}H\)DPCPX binding caused by adenine nucleotides. The addition of 2 units/ml ADA (third row) to the membrane preparation also reduced the displacement of \(^{3}H\)DPCPX binding by adenine nucleotides. Displacement of \(^{3}H\)DPCPX binding by adenosine and adenine nucleotides was minimal in the presence of both ADA and APCP (fourth row). This suggests that the displacement of \(^{3}H\)DPCPX binding by adenine nucleotides was due...
to adenosine formation from nucleotides during the 90-minute incubation period of the assay.

Figure 7B depicts the effects of various nucleotides and nucleosides (100 μM) on binding of [3H]DPCPX (2 nM) to bovine brain membranes. The A1-adenosine–selective agonist R-PIA (100 μM) was the only compound among those tested that potently and completely displaced [3H]DPCPX binding. The observed increase of binding in the presence of GTP has been previously reported.

The effect of various concentrations of AMP and AMPS, in the presence of ADA (2 units/ml) and APCP (100 μM), on [3H]DPCPX binding to bovine brain membranes is shown in Figure 8. A significant inhibition of binding was observed only at AMP and AMPS concentrations of 10 μM or more. This effect is negligible compared with the displacement caused by the A1-adenosine agonist CPA, which was almost complete at 1 μM.

Cardiac tissue. Binding of [3H]DPCPX to guinea pig cardiac membranes was significantly lower in the absence (Bmax = 4.5 fmol/mg protein) than in the presence (Bmax = 30 fmol/mg protein) of ADA, as determined in saturation binding experiments with

**Figure 7.** Panel A: Three-dimensional graph showing total [3H]DPCPX (2 nM) binding to bovine brain membranes in the presence of 100 μM of added adenine nucleotides and adenosine (ADO). Experiments were performed in the absence and presence of adenosine deaminase (ADA; 2 units/ml) and the 5′-nucleotidase inhibitor αβ-methylene-ADP (APCP; 100 μM). AMPS, adenosine monophosphorothioate. Panel B: Three-dimensional graph showing lack of inhibitory effect on total [3H]DPCPX (2 nM) binding of various nucleotides and nucleosides (initial concentration, 100 μM) to bovine brain membrane preparations incubated with ADA (2 units/ml). Note that (−)-N6-(2-phenylisopropy]l)adenosine (R-PIA) completely inhibited [3H]DPCPX binding. Data are the mean of three (for panel A) and two (for panel B) different experiments in duplicate.

**Figure 8.** Graph showing displacement of specific [3H]DPCPX (1 nM) binding by AMP, adenosine monophosphorothioate (AMPS), and N6-cyclopentyladenosine (CPA). Bovine brain membranes were incubated in the presence of 2 units/ml adenosine deaminase (ADA) and 100 μM αβ-methylene-ADP (except for CPA). The mean value for binding of [3H]DPCPX to membranes in the absence of competitor was normalized to 100% and is referred to as control. Data are expressed as mean ± SEM of three experiments in duplicate. **p<0.05 vs. control.
DPCPX (4 nM) were sine in menments (2 units/ml) deaminase no (panel 9). FIGURE 9 of for (except A3-10 0A3-10 nM 0A3-10 0A3-10 (61.9±6.4% ventricular adenosine and APCP units/ml) preparations. Brane may AMPS t10-100 nM 4 are in to atrial membranes, high AMPS uM) significantly inhibited binding of 4 nM [3H]DPCPX, despite the presence of ADA (2 units/ml) and APCP (100 μM). Similarly, 100 μM adenosine significantly inhibited [3H]DPCPX binding to both atrial (56.0±1.7% of control; p<0.01) and ventricular (61.9±6.4% of control; p<0.01) membranes in the presence of ADA (2 units/ml) and APCP (100 μM).

To further investigate whether the effect of AMP on binding is dependent on degradation of AMP to adenosine and other catabolites, guinea pig atrial membranes were preincubated for 30 minutes with rabbit antiserum against 5′-nucleotidase (1:100 dilution) before the binding experiment. ADA (2 units/ml) and APCP (100 μM) were also added to all incubation media. In antiserum-pretreated mem-

branes, AMP (100 μM) was slightly less effective in displacement of [3H]DPCPX binding than in membranes not pretreated with antiserum (55±3% [n=9] and 48±1.5% [n=6], respectively, of binding was displaced; p>0.05). Moreover, the combination of antiserum and 10 mM β-glycerophosphate, an inhibitor of nonspecific phosphatase,26 was not more effective than antiserum alone in reversing [3H]DPCPX displacement by AMP (57±3% [n=3] versus 55±3% [n=9], respectively). However, β-glycerophosphate alone attenuated the effect of 100 μM AMP (58±3% [n=3] versus 48±1.5% [n=6] with and without β-glycerophosphate, respectively; p<0.01).

The effect of AMP (100 μM) on [3H]DPCPX (4 nM) binding to guinea pig atrial membranes was significantly reduced in the presence of APCP (100 μM), ADA (2 units/ml), nucleoside phosphorylase (2 units/ml), and xanthine oxidase (1.5 units/ml), in comparison with the effect of AMP in the presence of ADA (2 units/ml) and APCP (100 μM) only (Figure 10). The results indicated that catabolites of adenosine (Figure 1) may displace [3H]DPCPX binding to guinea pig atrial membranes.

Therefore, additional competition experiments were carried out. Inosine at high concentration (100 μM) was able to compete with [3H]DPCPX (4 nM) for binding to guinea pig atrial membranes (57±3% of control; p<0.01 [Figure 11]), whereas hypoxanthine and xanthine displaced relatively little [3H]-DPCPX binding (90±4% [p<0.05 versus control] and 95±4% [p=NS versus control], respectively [Figure 11]). No displacement of [3H]DPCPX binding occurred in the presence of uric acid. Binding of [3H]DPCPX (1 nM) to bovine brain membranes (Figure 11, inset) was less affected by the adenosine metabolites than binding to atrial membranes.
Stability of AMP

The possibility that AMP is degraded during the 90-minute binding incubation period was investigated by measuring the recovery of AMP added to membrane preparations. As shown in Table 1, degradation of AMP (100 µM) after a 90-minute incubation with atrial membranes was nearly complete; degradation of AMP was less during incubation with membranes prepared from brain. AMPS was substantially degraded (but less completely than AMP) in both membrane preparations.

Discussion

The goal of this study was to test the hypothesis that adenine nucleotides can activate a specific potassium current, I\text{K}\text{ACh,Ado}, in atrial myocytes by binding to adenosine receptors. Our results are not consistent with this hypothesis. On the one hand, the actions of AMP on I\text{K}\text{ACh,Ado} and action potential duration in atrial myocytes were antagonized by 8-PST, a specific adenosine receptor antagonist. This result indicates that adenosine receptor stimulation mediated the electrophysiological action of AMP. On the other hand, the results of receptor binding experiments and electrophysiological studies with AMPS and with AMP in the presence of APCR and ADA are more consistent with the suggestion that adenine nucleotides must be degraded to adenosine before they can activate I\text{K}\text{ACh,Ado} in atrial myocytes.

ATP was approximately 10-fold less potent than AMP in shortening the atrial action potential and stimulating I\text{K}\text{ACh,Ado} (Figures 3 and 4). This result is expected in view of the increased number of enzymatic conversions needed to degrade ATP to adenosine. Activation of I\text{K}\text{ACh,Ado} by ATP binding to a P2-purinergic receptor was not excluded in this study, although there is no evidence that P2-purinergic receptors are linked to activation of I\text{K}\text{ACh,Ado} in atrial myocytes. Activation of the P2-purinergic receptor by ATP has been shown to increase calcium inward current in single isolated frog ventricular myocytes via a mechanism that involves phosphoinositide turnover.27 This action of ATP should not influence our results.

Inhibition of the degradation of AMP to adenosine and acceleration of the degradation of adenosine to inosine markedly reduced the effect of AMP on atrial cell electrophysiology. Also, the potencies and efficacies of both ATP and AMPS (which degrade more slowly than AMP to adenosine) in activating I\text{K}\text{ACh,Ado} and shortening the atrial action potential duration were less than those of AMP. The results suggest that the similar electrophysiological effects of adenine nucleotides and adenosine on atrial myocytes can be explained by enzymatic degradation of the former with concurrent accumulation of the latter. This conclusion is consistent with our previous study of the effects of ATP on atrioventricular node conduction in an isolated perfused guinea pig heart preparation. In that earlier study, it was found that the negative dromotropic action of ATP was dependent on a rapid degradation of ATP to adenosine.

**TABLE 1. Degradation of AMP and Adenosine Monophosphorothioate During Incubation in Guinea Pig Atrial and Bovine Brain Membrane Preparations**

| Membrane source | Recovery (%) | | | | |
|-----------------|--------------|---|---|---|
|                 | AMP          | AMPS | | | |
| Atria           | 3.1±0.9      | 35.1±0.4 | | | |
| Brain           | 39.4±6.0     | 57.6±2.9 | | | |

Data are mean±SEM of quadruplicate determinations from each of two experiments. Recovery of 100 µM AMP and 100 µM adenosine monophosphorothioate (AMPS) was determined after 90 minutes of incubation at 21°C with guinea pig atrial or bovine brain membranes, in the presence of αβ-methylene-ADP (100 µM) and adenosine deaminase (2 units/ml). AMP and AMPS concentrations were determined by high-performance liquid chromatography as described in "Materials and Methods." Protein content in samples was approximately 2.5 mg/ml for atria and approximately 0.5 mg/ml for brain.
Binding studies of nucleotides and nucleosides to cardiac and brain membranes support conclusions drawn from the electrophysiological studies. The relative affinity of adenosine nucleotides for the \(^{[3]H}\)DPCPX binding site, that is, the \(\text{A}_1\)-adenosine receptor, on membranes prepared from bovine brain and guinea pig heart tissues was determined by competition binding assays. It was found that adenosine nucleotides, especially AMP, did indeed displace \(^{[3]H}\)DPCPX binding to adenosine receptors. However, when precautions were taken to reduce the accumulation of adenosine and inosine during the 90-minute incubations of guinea pig atrial or bovine brain membranes with adenosine nucleotides and \(^{[3]H}\)DPCPX, displacement of specific \(^{[3]H}\)DPCPX binding was significantly reduced. Therefore, the most likely explanation for the apparent competition between AMP and \(^{[3]H}\)DPCPX for binding to \(\text{A}_1\)-adenosine receptors is displacement of \(^{[3]H}\)DPCPX by the adenosine and inosine that are formed when AMP is enzymatically degraded.

Substantial degradation of AMP and AMPS during incubation in guinea pig atrial and bovine brain preparations was observed in the present study (Table 1). Inhibition of 5’-nucleotidase with 100 \(\mu\)M APCP or with specific antiseraum appeared to inhibit AMP degradation only partially. The difficulty of inhibiting the degradation of AMP has been noted previously.\(^1\)\(^{15}\)\(^{26}\) Thus, the concentrations of nucleotides and their degradative products changed during the course of the incubation period of the receptor binding assays. In this circumstance the affinities of nucleotides and degradative products for the adenosine receptor cannot be distinguished. On the one hand, the affinity of AMP for the \(\text{A}_1\)-adenosine receptor may be underestimated because AMP concentration fell during incubation with membrane preparations; on the other hand, the observed effect of AMP on \(^{[3]H}\)DPCPX binding may be an artifact caused by adenosine and/or inosine.

To assess the effect of AMP per se on \(^{[3]H}\)DPCPX binding, ADA was used to remove the active product of AMP degradation, adenosine. ADA alone partially prevented displacement of \(^{[3]H}\)DPCPX binding to guinea pig atrial membranes by 100 \(\mu\)M AMP. Similarly, ADA reduced but did not abolish the displacement of \(^{[3]H}\)DPCPX binding by adenosine. Linden\(^28\) has observed that ADA is not able to completely remove adenosine present in membrane preparations. In addition, inosine that accumulates in high concentrations during incubations of membranes with adenosine and ADA may lead to displacement of \(^{[3]H}\)DPCPX binding. The confirmation of this suggestion came from the finding that 100 \(\mu\)M inosine caused a 45% reduction in \(^{[3]H}\)DPCPX binding to guinea pig atrial membranes, whereas hypoxanthine and xanthine caused less reduction of binding and uric acid had no effect (Figure 11). The binding activity of inosine is in agreement with data of Lohse et al\(^29\) for bovine myocardium. However, because inosine is devoid of any discernible electrophysiological effect, it appears that the binding of inosine to the \(\text{A}_1\)-adenosine receptor does not lead to receptor activation. In the presence of the enzymes ADA, nucleoside phosphorylase, and xanthine oxidase, which degrade adenosine, inosine, and hypoxanthine and xanthine, respectively (Figure 1), the AMP-induced displacement of \(^{[3]H}\)DPCPX binding was almost abolished (Figure 10). Therefore, we conclude that AMP appears to have a very low affinity for the \(^{[3]H}\)DPCPX binding site, that is, the \(\text{A}_1\)-adenosine receptor.

Altogether the results from both electrophysiological and binding studies are consistent with the hypothesis that adenosine nucleotides and purines, except for adenosine, do not bind and/or activate the cardiac \(\text{A}_1\)-adenosine receptor in atrial myocytes. This interpretation is limited by the fact that none of the approaches in the present study was completely successful in preventing degradation of adenosine nucleotides. The presence of a wide spectrum of adenosine nucleotide degradative enzymes in most commonly used cellular and membrane preparations poses serious difficulties to evaluate the “true” effects, not to mention the potency and efficacy, of adenosine nucleotide actions. Thus, the interpretation of physiological and pharmacological studies of adenosine nucleotides must be done with great caution.

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