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Interaction of Dihydropyridine Calcium Channel Agonists and Antagonists with Adenosine Receptors

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Abstract

We have confirmed our previous (Fredholm *et al.* 1986a) finding that the dihydropyridine calcium channel agonist Bay K 8644 can displace [³H]-R-PIA from its binding site, the adenosine A₁-receptor. Bay K 8644 had an apparent K_i of 5.2×10^{-6} M. The effect was shared by the two dihydropyridine calcium channel antagonists nifedipine and felodipine (K_i 4.2 and 8.7×10^{-6} M, respectively). By contrast, two non-dihydropyridine calcium channel antagonists, verapamil and diltiazem, did not affect binding. Bay K 8644 displaced [³H]-R-PIA from its binding sites in a solubilized preparation. [³H]-XAC, a novel, potent A₁-receptor antagonist ligand, was also displaced by the dihydropyridine compounds with a similar or slightly higher potency as the displacement of R-PIA. This suggests a direct interaction with the adenosine receptor rather than an effect on regulatory GTP-binding proteins. However, at 1 μ mol/l neither Bay K 8644 nor nifedipine significantly attenuated cyclic AMP accumulation in rat hippocampi or the R-PIA-mediated adenylate cyclase inhibition. The results show that dihydropyridine compounds that act as agonists or antagonists on L-type calcium channels can also affect adenosine receptors. The potency of the compounds for this effect is much lower than their potency as calcium channel agonists or antagonists. The results may therefore be of more experimental than clinical significance.

Adenosine inhibits the release of several transmitters in the peripheral as well as the central nervous system by an action on receptors of the A₁-subtype (cf. Fredholm & Hedqvist 1980; Fredholm *et al.* 1983; Dunwiddie & Fredholm 1984). The mechanism by which activation of adenosine A₁-receptors leads to inhibition of the release of the transmitters has been the subject of much research, but still remains enigmatic (for references see Dunwiddie & Fredholm 1985; Fredholm & Lindgren 1987). There is good recent evidence that a regulatory N- (or G)-protein, with similarities to the N_i-protein, is involved in the presynaptic effect of adenosine analogues (Dolphin & Prestwich 1985; Allgaier *et al.* 1985; Fredholm & Lindgren 1986 & 1987). Although such N-proteins are involved in the regulation of adenylate cyclase activity, there is no good evidence that the inhibition of transmitter release by adenosine is directly linked to changes in the activity of adenylate cyclase activity (Fredholm *et al.* 1983; Dunwiddie & Fredholm 1985; Fredholm & Lindgren 1986). Thus, it seems important to determine what other effector systems adenosine receptors may be linked to. Since depolarization-evoked transmitter release is strongly calcium-dependent, it seems natural to examine possible relationships between adenosine receptors and calcium channels.

We recently found that the so-called calcium channel agonist Bay K 8644 (Schramm *et al.* 1983), a dihydropyridine derivative related to e.g. nifedipine (see Snyder & Reynolds 1985), was able to antagonize certain effects of adenosine and also displace the binding of a labelled adenosine agonist, R-phenylisopropyl-adenosine (R-PIA) to its binding sites in the brain (Fredholm *et al.* 1986a). There are previous reports that the calcium channel antagonist nifedipine is able to displace the adenosine analogue cyclohexyl-adenosine (CHA) from its binding sites in the brain (Murphy & Snyder 1982), and that nifedipine may enhance certain actions of adenosine (Ribeiro *et al.* 1979; Phillis *et al.* 1984). Some of these effects may be due to the interaction with a nucleoside transporter, an interaction that shows a different stereospecificity from the interaction with the dihydropyridine binding site (Striessnig *et al.* 1985). These reports have led to the speculation that interactions between calcium channel antagonists and adenosine mechanisms may be of clinical significance (e.g. Swanson & Green 1986).

Based on these findings and considerations, we have examined the interaction of Bay K 8644 with the adenosine A₁-receptor. To examine effects on the binding to adenosine receptor we studied both the adenosine receptor agonist R-PIA and a recently discovered antagonistic xanthine amino congener, XAC (Jacobson *et al.* 1986). The effects of Bay K 8644 was compared to the effects of two dihydropyridine calcium entry blockers, nifedipine and felodipine, and to two non-dihydropyridine entry blockers, diltiazem and verapamil.

Materials and Methods

Preparation of rat brain membranes

Male Sprague-Dawley rats (weighing 150–250 g) were killed by decapitation and the brains rapidly dissected out. The brains were put in 8 volumes of 0.25 sucrose, 5 mM Tris-Cl, 2 mM MgCl₂, 1 mM EDTA, pH 7.4. After mincing with scissors, the tissue was homogenized in a glass-tenon homogenizer running at 400 r.p.m. for 10 strokes up and down. The homogenate was centrifuged at $2,800 \times g$ at 4° for 15 min. The supernatant was centrifuged again at $10,000 \times g$ for 15 min. and the resulting pellets were washed twice with the same buffer. The final wash was done in 100 mM Tris-acetate, 1.5 mM MgCl₂, 1 mM EGTA, pH 7.4 and then the pellets were suspended in 2 volume of buffer. The suspension was incubated 15 min. at 30° with 1 µg adenosine deaminase per ml and stored at –80°. The protein content was determined according to Lowry *et al.* (1951).

Due to the marked light-sensitivity of the dihydropyridines nifedipine and Bay K 8644 the experiments using these two compounds were carried out in the dark (shielding the experimental area by black plastic).

Solubilization of adenosine receptors

Rat brain membrane preparations described above were centrifuged at $10,000 \times g$ for 15 min. The pellets were suspended in 1% CHAPS, 100 mM Tris-acetate, 1.5 mM MgCl₂, 1 mM EGTA, pH 7.4 and homogenized with glass-teflon homogenizer with 3 or 4 strokes up and down (Nakata & Fujisawa 1984). The suspension was incubated at 0° for 30 min. and

centrifuged at $40,000 \times g$ for 1 hr. The supernatant (about 3–5 mg/ml protein) was stored at -80° until used.

Binding assay

To study binding of [3 H]-PIA to membrane receptors, 40–60 μ g protein and 2 nM radioligand were incubated in a total volume of 1 ml of 50 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4 at 30° for 2 hrs. The assay was terminated by filtration through Whatman GF/B glass-fiber filters, soaked for 1 hr in cold buffer, and washed two times with 5 ml ice-cold incubation buffer. The non-specific binding was determined in the presence of 1 μ M CHA. The binding of [3]-XAC was studied in almost the same way except that the filters were soaked in 0.3% polyethylenimine (PEI) for at least 1 hr before use to diminish the non-specific binding as described previously (Bruns *et al.* 1983; Jacobson *et al.* 1986). The non-specific binding was determined in the presence of 10 μ M DPX.

The [3 H]-R-PIA binding to soluble receptors was measured essentially as described (Stiles 1985). One ml aliquots containing 0.1–0.3 mg protein, 2 nM [3 H]-R-PIA, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4 were incubated for 90 min. at 25° . Two hundred μ l of incubation mixture was applied to a Sephadex G-50 column (0.7×12 cm), which had been preequilibrated with 0.1% CHAPS, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4. After eluting 1 ml of void volume, the next 1.5 ml was directly collected into vials. The columns were washed with 20 ml of the buffer and used again. The non-specific binding was determined in the presence of 1 μ M CHA.

Accumulation of [3 H]-cyclic AMP

The conversion of [3 H]-adenine-labelled nucleotide stores into cyclic AMP was measured as described in detail elsewhere (Fredholm *et al.* 1982). The slices were usually treated with drugs for a period of 15 min. At the end of the incubation period they were homogenized in 0.4 M perchloric acid which was neutralized. The protein free supernatant after centrifugation was used to determine the total radioactivity and the remainder was put on combined Dowex and alumina chromatography to separate [3 H] cyclic AMP from [3 H]-ATP and other adenine nucleotides. The results are expressed as per cent of the total radioactivity present in the slice that was converted into cyclic AMP. Corrections are made for incomplete recovery of [3 H]-cyclic AMP over the chromatography steps.

Drugs

The following drugs and reagents were used: [2- 3 H]-adenine (25 Ci/mmol) and [8- 3 H]-adenosine 3', 5'-monophosphate, ammonium salt (28 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, UK. [3 H]-xanthine amine congener of 1,3-dipropyl-8-phenylxanthine, 8-(4'-carboxymethyloxyphenyl)-1,3-dipropylxanthine-2-aminoethylamide, was synthesized as described (Jacobson *et al.* 1986). [3 H]-PIA, ((-)-N6-R-[3 H]phenylisopropyladenosine, 1.48 TBq/mmol, 40 Ci/mmol), Radiochemical Centre, Amersham. Methyl 2,6-dimethyl-5-nitro-4-(2-trifluoromethylphenyl) 1,4-dihydropyridine-3-carboxylate, Bay K 8644, and nifedipine were from Bayer AG. Adenosine, adenosine 3',5'-monophosphate, verapamil were obtained from Sigma, St. Louis, MO, U.S.A. Forskolin and 8-phenyltheophylline were obtained from Calbiochem-Behring, La Jolla, CA, U.S.A. GTP

and R-PIA were obtained from Boehringer, Mannheim, FRG, Rolipram (ZK 62,711, 4-(3-cyclopentylloxymethoxyphenyl)-2-pyrrolidone) was a gift from Schering AG, Berlin, FRG. Felodipine was a gift from AB Hässle, Mölndal, Sweden, and diltiazem from Tanabe, Japan.

Results

The binding of [3 H]-R-PIA and [3 H]-XAC to A_1 -receptors was essentially as described earlier (Jacobson *et al.* 1986; Stiles 1985; Schwabe 1985). The binding parameters for [3 H]-R-PIA were K_D $2.7 (2.0\text{--}4.2) \times 10^{-9}$ M; B_{max} 478 (246–1053) fmol/mg protein. The K_D and B_{max} for [3 H]-XAC were 1.48×10^{-9} M and 535 fmol/mg protein (fig. 1).

The [3 H]-R-PIA binding to A_1 receptors was dose-dependently inhibited by Bay K 8644. (IC_{50} $7.19 \pm 1.8 \times 10^{-6}$ M; fig. 2). The degree of inhibition by Bay K 8644 was very similar when instead a soluble receptor preparation was used (IC_{50} 5.65×10^{-6} M). The guanine nucleotides can inhibit the binding of adenosine agonists but not antagonists to A_1 -receptors by acting on the N_i -protein (Cooper *et al.* 1980; Jacobson *et al.* 1986). In the presence of 50 μ mol/l GTP the binding of [3 H]-R-PIA was reduced by about 10 per cent in a membrane preparation but almost completely in a soluble preparation (not shown). This is in agreement with the results of Stiles (1985). There was no clearcut interaction between Bay K 8644 and GTP, but such possible interactions were not examined in detail. We further examined the effect of the dihydropyridine calcium channel agonist on the binding of an antagonist, [3 H]-XAC. Bay K 8644 displaced the [3 H]-XAC binding to A_1 -receptor (IC_{50} $2.96 \pm 0.72 \times 10^{-6}$ M, fig. 2) at least as well as the binding of [3 H]-PIA.

In order to examine if the effect of Bay K 8644 was unique, we also examined the effects of nifedipine and felodipine, two dihydropyridine calcium channel antagonists (Ljung 1985). The two drugs inhibited both [3 H]-R-PIA and [3 H]-XAC binding to A_1 -receptors (figs. 3 & 4). By contrast, two calcium antagonist that are not dihydropyridines, verapamil and diltiazem, were virtually ineffective.

Effect of Bay K 8644 on 3 H-cAMP accumulation in hippocampal slices

In order to examine if the inhibition by Bay K 8644 of the binding of an adenosine analogue could explain our previous finding that 1 μ mol/l Bay K 8644 blocked the presynaptic effects of R-PIA we examined the effect of Bay K 8644 on cyclic AMP accumulation in the rat hippocampus. In this tissue it is possible to study both A_1 - and A_2 -receptor mediated effects by following cyclic AMP (Fredholm *et al.* 1986b). Low doses of R-PIA are able to reduce the accumulation of cyclic AMP afforded by forskolin. As shown in table 1, neither Bay K 8644 nor nifedipine materially altered the cyclic AMP accumulation afforded by forskolin or the inhibitory effect of R-PIA.

Discussion

The binding of [3 H]-PIA and [3 H]-XAC to adenosine receptors was inhibited by dihydropyridine derivatives irrespective of whether they are calcium channel agonists or antagonists. It is therefore unlikely that the effect of the dihydropyridine derivatives is related to their activating or inactivating the calcium channel. This conclusion is further

supported by the finding that the two structurally unrelated calcium channel antagonists, diltiazem and verapamil, did not affect binding the adenosine receptor (Murphy & Snyder 1982).

Bay K 8644 was effective not only in membranes, but also in a solubilized preparation. The binding of [3 H]-PIA to both membrane and soluble receptors was reduced by 10 μ M of GTP. The binding to the soluble receptor was almost completely blocked by 50 μ mol/l GTP, but the effect was much smaller on binding of R-PIA to membrane receptors. Besides the obvious possibility that the GTP-hydrolysis is lower in solubilized than in intact membrane preparations, there are two possible explanations for this finding: First, the linkage of receptors to N_i -protein may be much looser after solubilization and hence influenced more easily by nucleotide and other factors; second, there may be an unknown factor in membrane bound proteins that is destroyed or lost by solubilization and that serves to regulate the binding of A_1 -receptor to N_i -protein (Stiles 1985).

Since 3 H-XAC is an antagonist, it binds to the receptor in a way that is virtually uninfluenced by GTP, and the receptor in its antagonist-binding conformation does not seem to interact with regulatory N-proteins (Jacobson *et al.* 1986; Schwabe 1985). Therefore, the present finding that the [3 H]-XAC-binding was influenced by dihydropyridines in the same way as was [3 H]-R-PIA-binding provides good evidence that the dihydropyridines bind to the adenosine receptor rather than to a regulatory GTP-binding protein associated with the receptor.

There appears to be several types of binding sites for dihydropyridines in rat brain membranes: a high affinity dihydropyridine-binding site that is linked to calcium channels (Ramkumar & El-Fakahany 1986), and low affinity sites linked to a purine uptake site (Striessnig *et al.* 1985) and another low affinity site associated with adenosine receptors (Murphy & Snyder 1982, and our results). We do not know what the relationship between the different kinds of binding sites for dihydropyridines derivatives may be. We have shown that Bay K 8644 blocks the effect of an adenosine agonist on transmitter release (Fredholm *et al.* 1986a). Furthermore, Bay K 8644 reduced the potency of adenosine in inhibiting the electrically evoked contraction of the rat vas deferens (Lee 1985). It is therefore important to consider the possibility that Bay K 8644 interferes with the effects of adenosine by virtue of its effects on the adenosine receptor and not because it interacts with the calcium channel.

Bay K 8644 is known to bind to dihydropyridine receptors with an apparent affinity close to 30 nM (Belleman 1984). It activates calcium channels, and increases transmitter release (Middlemiss & Spedding 1985) and inositol phosphate turnover (Kendall & Nahorski 1985) in the same submicromolar concentrations. On the other hand, the potency of Bay K 8644 to inhibit adenosine receptor binding was in the μ molar range. In the present study we show that Bay K 8644 and nifedipine, in concentrations that do alter adenosine receptor mediated transmitter release, produce very small effects on the adenosine receptor mediated inhibition of forskolin stimulated cyclic AMP accumulation, an adenosine A_1 -receptor mediated response. We have found that even though the R-PIA mediated inhibition of NA release induced by stimulation is blocked by Bay K 8644 (Fredholm *et al.* 1986a) the effect of R-PIA on the release of NA induced by potassium-depolarization is not (Swanson, Lindgren &

Fredholm, unpublished results) nor is that on the release of acetylcholine (Dunne-r-Engström & Fredholm, unpublished results). We also show here that the dihydropyridine compounds have the same displacing effect irrespective of whether they are agonists or antagonists. By contrast, the biological effects were seen only by the agonist and the effect of Bay K 8644 was blocked by the antagonists (Lee 1985; Fredholm *et al.* 1986a). For all these reasons we are reluctant to ascribe the interactions between adenosine and Bay K 8644 described before to an interaction at the adenosine A₁-receptor.

In conclusion, we have confirmed previous findings (see Murphy & Snyder 1982) that various dihydropyridine compounds are able to interact with the binding of adenosine receptor agonists to A₁-receptor sites in the brain. We have confirmed and somewhat extended these findings and also provided evidence that this biochemical effect is probably not the cause of the antagonism by Bay K 8644 of the presynaptic actions of R-PIA that can sometimes be observed. Even so the present results indicate that high concentrations of the dihydropyridine compounds may, under experimental conditions, have effects that are related to an effect on adenosine receptors or, as shown elsewhere (Striessnig *et al.* 1985), adenosine uptake, rather than to an interaction with calcium channels.

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Fig. 1. Binding of [^3H]-XAC to rat cortical membrane adenosine receptors. A. Specific binding of [^3H]-XAC (1–10 nmol/l) to membrane receptors. Means of assays in duplicate or triplicate. 40 mg membrane protein incubated for 2 hrs at 37° in a total volume of 1 ml. B. Scatchard plot of the same data.

Fig. 2.

The effect of Bay K 8644 on binding of [3 H]-R-PIA and [3 H]-XAC to membrane and soluble receptors. Filled circles represent displacement of R-PIA bound to membrane receptors (K_i app. $5.23 \pm 1.22 \times 10^{-6}$, B_{max} app. 425 fmol/mg); Triangles represent displacement by Bay K 8644 of [3 H]-XAC bound to membrane receptors (K_i app. $1.46 \pm 0.28 \times 10^{-6}$, B_{max} app. 897 fmol/mg protein). Triangles represent displacement by Bay K 8644 of [3 H]-R-PIA bound to soluble receptors (K_i app. 1.52×10^{-6} , B_{max} app. 168 fmol/mg protein). Results are means and s.e.m. of triplicate determinations from 2–3 separate experiments. The curves are derived from computer analysis (SCAFIT) of the data.

Fig. 3. Displacement by nifedipine of $[^3\text{H}]\text{-R-PIA}$ and $[^3\text{H}]\text{-XAC}$ bound to membrane receptors. Open symbols $[^3\text{H}]\text{-R-PIA}$ binding (K_i app. $4.15 \pm 1.37 \times 10^{-6}$, $B_{\max}$ app. 653 fmol/mg protein); closed symbols $[^3\text{H}]\text{-XAC}$ (K_i app. $1.86 \pm 0.42 \times 10^{-6}$ M, $B_{\max}$ app. 868 fmol/mg protein).

Fig. 4. Displacement of [3 H]-R-PIA and [3 H]-XAC bound to membrane receptors by felodipine, but not by verapamil and diltiazem. The apparent K_i app. for felodipine $8.71 \pm 0.76 \times 10^{-6}$ M for R-PIA and 3.04 ± 10^{-6} M for XAC. Further details see text to fig. 2.

Table 1

Effect of Bay K 8644 and nifedipine on ^3H -cyclic AMP accumulation in slices of rat hippocampus. Results are mean and S.E.M. Number of determinations within parenthesis.

	^3H -cyclic AMP accumulation (% of total ^3H)		
	-R-PIA	1 μM R-PIA	% inhib.
Forskolin 1 μM	2.04 $\pm$ 0.23 (9)	1.77 $\pm$ 0.25 (9)	15
	P < 0.05		
Forskolin+Bay K 1 μM	1.89 $\pm$ 0.10 (9)	1.58 $\pm$ 0.12 (9)	16
	P < 0.05		
Forskolin+Nifedipine 1 μM	1.95 $\pm$ 0.22 (9)	1.63 $\pm$ 0.18 (6)	22
	P < 0.05		