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INHIBITION OF RADIOLIGAND BINDING TO A_1 ADENOSINE RECEPTORS BY BAY K8644 AND NIFEDIPINE

WING-TAI CHEUNG, MAGGIE M. SHI, JAMES D. YOUNG and CHI-MING LEE*
Department of Biochemistry, Faculty of Medicine, Chinese University of Hong Kong, Hong Kong

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Abstract—Two dihydropyridine compounds, Bay K8644 (a calcium entry activator) and nifedipine (a calcium entry blocker), were found to inhibit the binding of [^{3}H]phenylisopropyladenosine ([^{3}H]PIA) to A_1 adenosine receptors in rat cerebral cortex membranes with comparable potencies (IC_{50} 10–30 μM). Scatchard analyses indicated that both Bay K8644 and nifedipine inhibited the binding of [^{3}H]PIA by increasing the K_D but without significant effect on the B_{max}. When tested at 100 μM, neither Bay K8644 nor nifedipine showed a significant effect on [^{3}H]-*p*-aminoclonidine ([^{3}H]PAC; α_2-adrenergic receptor), [^{3}H]dihydroalprenolol ([^{3}H]DHA; β-adrenergic receptor), [^{3}H]spiperone (dopamine receptor), and [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR; nucleoside transporter) binding. In the presence of 10 mM Mg^{2+}, the ability of 2-chloroadenosine (2-Cl-Ad, an A_1 adenosine receptor agonist) to displace [^{3}H]PIA binding was increased. Conversely, the potencies of 1,3-diethyl-8-phenylxanthine (DPX; an A_1 receptor antagonist), Bay K8644 and nifedipine in inhibiting [^{3}H]PIA binding were unchanged. It is suggested that both Bay K8644 and nifedipine may act as antagonists of adenosine A_1 receptors, in addition to their well-known effects on calcium channels.

Some dihydropyridines, including nifedipine, nitrendipine and nimodipine, exert potent antagonistic effects on calcium-dependent processes such as hormone secretion from endocrine cells and contraction of smooth and cardiac muscle [1–3]. Results of radioreceptor analyses suggest a correspondence of the high-affinity dihydropyridine binding site with the voltage-sensitive calcium channel, and many biological effects of dihydropyridines may be attributed to their effect on these calcium channels [4]. More recently, Bay K8644 [methyl-1,4-dihydro-2,6-dimethyl- 3- nitro- 4- (2- trifluoromethylphenyl)- pyridine-5-carboxylate], a structural analogue of nifedipine, was reported to facilitate the entry of calcium into smooth and cardiac muscle [5], and both Bay K8644 and nifedipine appear to share a common high-affinity binding site [6].

Adenosine is a putative neurotransmitter in the central and peripheral nervous system [7, 8]. It influences many biological processes including sedation, inhibition of platelet aggregation, bronchoconstriction and vasodilation [9]. It has been suggested that adenosine may exert its effect by modulating the transmembrane calcium flux into nerve terminals and/or smooth muscle [10, 11]. We have demonstrated recently that adenosine can inhibit the electrically evoked contractions of the rat vas deferens via the activation of A_1 adenosine receptors [12]. Furthermore, Bay K8644 can reduce the inhibitory potency of adenosine in the rat vas deferens in a calcium-dependent manner [13]. While Bay K8644 may antagonize the adenosine inhibition by facilitating calcium entry in the vas deferens, a direct antagonistic action at the adenosine A_1 receptor cannot be ruled out. To differentiate between these two mechanisms, the effects of Bay K8644 and nifedipine on [^{3}H]phenylisopropyladenosine ([^{3}H]PIA) binding to A_1 adenosine receptors were examined in the present study.

* Send correspondence to: Dr. C-M. Lee, Department of Biochemistry, Faculty of Medicine, Chinese University of Hong Kong, Shatin, N.T., Hong Kong.

MATERIALS AND METHODS

Male Sprague–Dawley rats (300–350 g) were killed by cervical dislocation. Cerebral cortex was homogenized in 10 vol. (w/v) of ice-cold 50 mM Tris–HCl buffer (pH 7.4 at 22°) using a Brinkmann Polytron PT-10 (setting 6, 10–15 sec). The homogenate was centrifuged at 45,000 *g* for 10 min, and the pellet was washed three times in 20 vol. of the same buffer. The crude membrane fraction was stored as a pellet at −20°. For adenosine A_1 receptor binding assays, the membrane pellet was resuspended in the same Tris–HCl buffer to give a protein concentration of about 1 mg/ml, and 350 μl of the membrane preparation was incubated with [^{3}H]PIA at indicated concentrations in duplicate. The incubation was carried out in a total volume of 500 μl for 1 hr at 37° that contained 50 mM Tris–HCl (pH 7.4 at 22°), 0.5 units/ml adenosine deaminase (EC 3.5.4.4) and various tested drugs. The reaction was terminated by filtration on a glass fibre filter (Whatman GF/B) under suction. The filters were washed four times with 3-ml aliquots of ice-cold Tris–HCl buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence of 100 μM 2-chloroadenosine (2-Cl-Ad). Specific binding was calculated by subtracting the non-specific binding from total binding. The duplicates varied less

Table 1. Inhibition of [^{3}H]PIA binding to rat cerebral cortex membranes

Drugs*	Control		10 mM Mg^{2+}	
	IC_{50}† (μM)	Hill coefficient	IC_{50} (μM)	Hill coefficient
2-Cl-Ad	0.05 ± 0.004	1.09 ± 0.05	0.03 ± 0.001‡	0.90 ± 0.03
DPX	0.48 ± 0.10	0.94 ± 0.08	0.47 ± 0.01	0.92 ± 0.06
Nifedipine	26.33 ± 5.33	0.74 ± 0.05	24.00 ± 3.51	0.83 ± 0.01
Bay K8644	14.33 ± 1.45	1.08 ± 0.10	15.67 ± 0.33	0.99 ± 0.04

* Bay K8644 and nifedipine were freshly prepared in absolute ethanol to give a 10 mM stock solution and protected from light. 2-Cl-Ad and DPX were also freshly prepared in absolute ethanol to give 10 mM and 1 mM stock solutions respectively. The final concentration of ethanol was 3% (v/v) in the [^{3}H]PIA binding assay.

† IC_{50} is the concentration of drug required to inhibit specific binding of [^{3}H]PIA (2 nM) by 50%. Values are mean ± SEM of three separate experiments performed in duplicate.

‡ $P < 0.005$, indicating a significant difference between the IC_{50} values determined in the presence and absence of 10 mM Mg^{2+} by Student's *t*-test.

than 10%. Protein was determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard. Other radioligand binding assays were performed as described previously: [^{3}H]-*p*-aminoclonidine ([^{3}H]PAC) for the α_2-adrenergic receptor by Rouot and Snyder [15], [^{3}H]dihydroalprenolol ([^{3}H]DHA) for the β-adrenergic receptor by Bylund and Snyder [16], [^{3}H]spiperone for the dopamine receptor by Creese *et al.* [17], and [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) for the nucleoside transporter by Shi *et al.* [18]. [^{3}H]Spiperone labels both dopamine and serotonin ($5HT_2$) receptors. To avoid interference from $5HT_2$ receptors in the frontal cortex, rat striatal tissues were used for the study of dopamine receptors. All the other radioreceptor assays were performed on rat cerebral cortical membranes except [^{3}H]NBMPR binding, the numbers of nucleoside transporters in rat cerebral cortex being low (M. M. Shi and J. D. Young, unpublished data). To maximize the signal, rat lung membrane was used for the [^{3}H]NBMPR binding study. Each binding experiment was repeated at least three times.

[^{3}H]PAC was purchased from New England Nuclear (Boston, MA). [^{3}H]Spiperone and [^{3}H]PIA were from Amersham International plc (Buckinghamshire, England). [^{3}H]NBMPR was from Moravek Biochemicals Inc. (Brea, CA). Nifedipine, nitrobenzylthioguanosine (NBTGR), 2-Cl-Ad, dopamine, norepinephrine (NE) and isoproterenol (ISO) were from the Sigma Chemical Co. (St. Louis, MO). 1,3-Diethyl-8-phenylxanthine (DPX) was obtained from Research Biochemical Inc. (Wayland, MA), and Bay K8644 was a gift from Bayer (Wuppertal, F.R.G.).

RESULTS

Both Bay K8644 and nifedipine inhibited the binding of [^{3}H]PIA to A_1 adenosine receptors with IC_{50} values (concentration required to inhibit specific binding by 50%) of 14 and 26 μM respectively (Table 1). They were less potent when compared to 2-Cl-Ad and DPX which displayed IC_{50} values of 0.05 and 0.48 μM respectively. In the presence of 10 mM Mg^{2+}, the IC_{50} value of 2-Cl-Ad was decreased by about 2-fold, whereas those of DPX, nifedipine and Bay K8644 were unchanged (Table 1). The displacement of [^{3}H]PIA binding by the various tested drugs appeared to be parallel and monophasic with a pseudo-Hill coefficient of about one, in both the presence and absence of 10 mM Mg^{2+}.

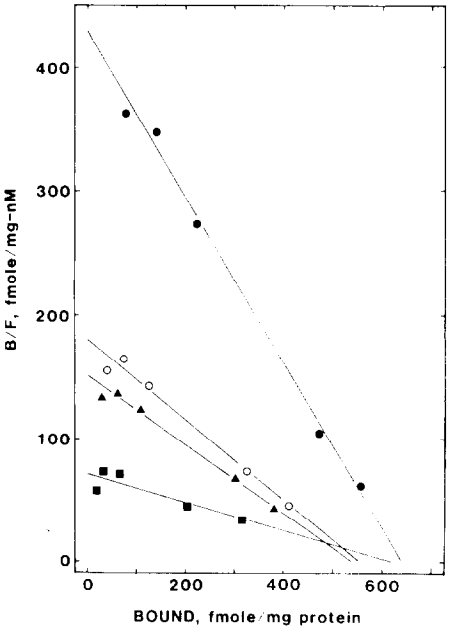

Fig. 1. Scatchard plots of [^{3}H]PIA (0.1 to 10 nM) binding to rat cerebral cortex membranes in control (●), 200 nM DPX (○), 5 μM Bay K8644 (■) and 5 μM nifedipine (▲). The straight lines were determined by linear regression analyses. Data shown are those of a typical experiment. Similar results were obtained in two additional experiments performed in duplicate.

The Scatchard analysis of the concentration dependence of specific [^{3}H]PIA binding indicated that the [^{3}H]PIA binding site was homogenous with an apparent K_D of 1.5 nM and a B_{max} of 640 fmol/mg protein (Fig. 1). In the presence of 200 nM DPX, the affinity of [^{3}H]PIA binding to the adenosine A_1 receptor was decreased about 6-fold (K_D = 8.7 nM), but there was little or no change in the B_{max} (620 fmol/mg protein). Similar to DPX, the presence of 5 μM nifedipine and

Table 2. Effects of 100 μM nifedipine and Bay K8644 in radioligand binding assays

^{3}H-Labeled ligand	Radioligand concentration (nM)	Binding (% of control)	
		Nifedipine	Bay K8644
[^{3}H]Spiperone	1.0	92 ± 5 (3)	87 ± 5 (3)
[^{3}H]DHA	1.0	90 ± 1 (3)	85 ± 15 (3)
[^{3}H]PAC	1.0	88 ± 15 (4)	90 ± 9 (4)
[^{3}H]NBMPR	0.5	87 ± 2 (3)	82 ± 3 (3)

Nifedipine and Bay K8644 were prepared as described in Table 1. Values presented are the means ± SD of (N) separate experiments performed in duplicate. Non-specific binding was defined as follows: [^{3}H]spiperone, 500 μM dopamine; [^{3}H]PAC, 100 μM norepinephrine; [^{3}H]DHA, 100 μM isoproterenol; and [^{3}H]NBMPR, 20 μM nitrobenzylthioguanosine. The binding of [^{3}H]spiperone binding to dopamine receptors was determined with rat striatal membranes. The binding of [^{3}H]DHA and [^{3}H]PAC to β- and α_2-adrenergic receptors, respectively, was determined with rat cerebral cortical membranes, whereas [^{3}H]NBMPR binding to nucleoside transporters was determined with rat lung membranes.

Bay K8644 caused a 2-fold decrease in affinity (K_D = 3.6 and 3.1 nM respectively). On the other hand, both nifedipine and Bay K8644 caused a slight but statistically insignificant decrease in the B_{max} (540 and 554 fmol/mg protein respectively).

When tested at 100 μM, neither Bay K8644 nor nifedipine had a significant effect on the binding of [^{3}H]PAC (α_2-adrenergic receptor) and [^{3}H]DHA (β-adrenergic receptor) to rat cerebral cortex membranes (Table 2). Similarly, there was little effect on the binding of [^{3}H]spiperone (dopamine receptor) to rat striatal membranes or on the binding of [^{3}H]NBMPR (nucleoside transporter) to rat lung membranes (Table 2).

DISCUSSION

An inhibitory effect of nifedipine on [^{3}H]cyclohexyladenosine binding has been reported previously [19]. The present study shows that, in addition to nifedipine, its structural analogue, Bay K8644, can also inhibit [^{3}H]PIA binding to rat cerebral cortex membranes. Preliminary studies indicate that Bay K8644 and nifedipine can also inhibit [^{3}H]PIA binding to adenosine receptors in rat brain striatal membranes and fat cell membranes with similar potencies (W-T. Cheung and C-M. Lee, unpublished data). To evaluate whether Bay K8644 (a calcium entry activator) and nifedipine (a calcium entry blocker) behave as agonists or as antagonists at the adenosine A_1 receptor, we examined [^{3}H]PIA binding in the presence of Mg^{2+}, which has been shown previously [20] to enhance agonist binding while leaving antagonist binding unchanged. In agreement with the observation of Goodman *et al.* [20], the presence of 10 mM Mg^{2+} was found to increase 2-Cl-Ad (agonist) potency but did not affect DPX (antagonist) potency in inhibiting [^{3}H]PIA binding to adenosine A_1 receptors (Table 1). Furthermore, the presence of 10 mM Mg^{2+} did not change the IC_{50} of either Bay K8644 or nifedipine, suggesting that both Bay K8644 and nifedipine may act as adenosine A_1 receptor antagonists (Table 1). This may account in part, if not entirely, for the antagonistic action of Bay K8644 against adenosine inhibition in the rat vas deferens [12], as well as the antagonistic action of nifedipine against adenosine elevation of dog coronary blood flow [21]. The lack of antagonism of nifedipine (10^{-7}–10^{-9} M) on adenosine-mediated relaxation of femoral [22] and coronary [23] arteries may be accounted for by the low concentration of nifedipine employed in these studies.

Nifedipine has been reported to inhibit [^{3}H]NBMPR binding to nucleoside transporters from human erythrocytes [24], guinea pig cardiac membranes [24], dog heart and forebrain membranes [25], as well as to inhibit adenosine uptake by rat brain cortical synaptosomes [26]. In the case of human erythrocytes and guinea pig cardiac membranes, reported apparent K_i values for nifedipine inhibition of [^{3}H]NBMPR binding are in the high micromolar range (82 and 50 μM respectively) [24]. In agreement with these results, we found that nifedipine and Bay K8644 were relatively ineffective inhibitors of [^{3}H]NBMPR binding to rat lung membranes (Table 2).

In accordance with previous reports [19, 27], nifedipine was found not to affect the binding of [^{3}H]PAC and [^{3}H]DHA to adrenergic receptors. We have extended these observations to Bay K8644 and obtained similar results (Table 2). Thus, the inhibitor effects of Bay K8644 and nifedipine on the adenosine A_1 receptor, although not very potent, are rather selective. In conclusion, in spite of the very potent action of nifedipine and Bay K8644 on the calcium channel, it is important to note that these dihydropyridines, particularly at high concentrations, may also exert their effects by acting as adenosine receptor antagonists.

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