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BINDING OF A 1,4-DIHYDROPYRIDINE CALCIUM CHANNEL ACTIVATOR, (-) S BAY K 8644, TO CARDIAC PREPARATIONS

J. Ferrante, E. Luchowski, A. Rutledge and D.J. Triggle

Department of Biochemical Pharmacology,

School of Pharmacy,

State University of New York, Buffalo, New York, 14260

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SUMMARY The 1,4-dihydropyridine Ca2+ channel activator, (-) [^{3}H]Bay K 8644, binds to cardiac membranes and polarized [5 mM K+] and depolarized [50 mM K+] cardiac cells. Binding to microsomal membranes at 25°C indicates a single set of binding sites, K_D = 2.9 x 10^{-9}M and a site density, 337 fmoles/mg protein, not different from that measured by antagonist 1,4-dihydropyridines. Binding to neonatal rat myocytes at 37°C was independent of membrane potential with a K_D value of 5 x 10^{-8}M and a site density, 63 fmoles/mg protein, not significantly different from that measured by PN 200 110. These results indicate that 1,4-dihydropyridine activators and antagonists label the same number of binding sites in cardiac tissue, but that activator binding to intact myocytes is voltage-independent.

The 1,4-dihydropyridine structure represents both antagonists and activators potently active at the L class of voltage-dependent Ca2+ channel [for reviews see 1 - 3]. Radioligand binding studies with 1,4-dihydropyridine antagonists have been widely employed in the definition of the sites and mechanisms of action of Ca^{2+} channel ligands. Absolute and relative correlations between binding and pharmacologic activities support the hypothesis that the high affinity binding sites measured in membrane preparations mediate the observed pharmacologic effects [2,4,5]. However, the voltage-dependence of 1,4-dihydropyridine interactions limits comparison to the inactivated channel state presumably dominant in membrane preparations [2,6,7].

In contrast, few studies are available that measure directly the binding of 1,4-dihydropyridine activators and these have employed racemic [^{3}H]Bay K 8644 in membrane preparations [5,8-10]. A major limitation to such studies is that the S and R enantiomers of Bay K 8644 exhibit the opposing properties of channel activation and antagonism respectively [11,12]. Binding studies with the racemic

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compound do not differentiate these properties and hence comparisons of discrete 1,4-dihydropyridine activator and antagonist interactions are impossible in such membrane systems.

In the present work we have investigated the specific binding of the S enantiomer of Bay K 8644 to cardiac membrane fragments and to cultured cardiac cells under polarized and depolarized conditions. Under polarized to modestly depolarized conditions (-) S Bay K 8644 behaves as a Ca^{2+} channel activator, prolonging the channel open state [13]. A comparison is provided to the binding of the antagonist 1,4-dihydropyridines (+) [^{3}H]PN 200 110 and (+) [^{3}H]nitrendipine.

METHODS

Cardiac Membranes. Microsomal membranes were prepared as previously described [14] from the hearts of male guinea pigs [Buckberg Farms, Tomkins Cove, NY] of weight 350 - 450g.

Cultured Cardiac Cells. Ventricular cardiomyocytes were prepared in primary culture by a combination of reported methods [15,16]. In brief, ventricles from 5 day neonatal rats were placed in a Ca^{2+} and Mg^{2+} free saline of the following composition [mM]: NaCl, 127; KCl, 4.56; KH_2PO_4, 0.44; $NaHCO_3$, 4.16; NaH_2PO_4, 0.63; glucose, 5.56 and Hepes, 20 at pH 7.4. The ventricles were minced and dispersed by trypsinization and the dispersion plated at a density of 0.5 million cell per ml in Corning 35 mm plastic dishes [2 ml per dish] in minimum essential medium [MEM, with Earle's salt] 85%, horse serum 15%, Hepes 25mM, L-glutamine 4 mM, penicillin 100 units/ml, streptomycin 100 µg/ml and amphotericin b 250 ng/ml maintained under 5% CO_2 and 95% air at 37^{o}C. Cells were used at 5 to 7 days of culture.

Radioligand Binding. Binding to membrane fragments was carried out as previously described with 5 ml assay volumes at a protein density of 20 - 30 µg/ml [14]. For cardiac membranes 50 mM Tris buffer, pH 7.2 at 25^o was employed for both [^{3}H]Bay K 8644 and [^{3}H]PN 200 110 binding with incubation periods of 30 to 90 mins. Nonspecific binding was determined by 10^{-6}M unlabelled ligand. Binding was terminated by filtration over GF/B filters followed by three washes with 50 mM Tris buffer at 0^{o}C [5 ml]. Whole cell binding [16] was carried out with cells attached to the culture dishes using medium containing either 5 mM KCl [polarized] or 50 mM KCl [depolarized] with incubation times of 30 - 90 min for [^{3}H]Bay K 8644 and [^{3}H]PN 200 110. Protein was measured using bovine serum albumin as standard [17]. Stock solutions of drugs were prepared in ethanol and the highest concentration achieved in the assay, 0.5%, was without effect on the measured specific binding.

(-) S [^{3}H]Bay K 8644 and (+) [^{3}H] PN 200 110, with specific activities of 85 and 86 Ci/mmole respectively, were obtained from Du Pont-New England Nuclear, Boston, Ma. (-) S[^{3}H]Bay K 8644 was obtained through the courtesy of Bayer AG, Wuppertal, F.R.G. The R and S enantiomers of Bay K 8644 were provided by Dr. A. Scriabine, Miles Institute for Preclinical Pharmacology, West Haven, CT.

RESULTS AND DISCUSSION

(-) S Bay K 8644 binds in saturable manner to a microsomal membrane fraction from rat heart ventricles [Figure 1]. Over the concentration range of Bay K 8644 studied, 2 x 10^{-10} to 10^{-8}M, specific binding appeared to be to a single set of saturable binding sites with a K_D value of 2.9 ± 0.5 x 10^{-9}M and a maximum binding capacity of 337 ± 69 fmoles/mg protein [n = 7; Table 1]. Specific binding, defined by 10^{-6}M Bay K 8644, represented 30 to 65% of the total bound ligand under the conditions employed. Specific binding was not significantly different whether defined by Bay K 8644 , nitrendipine or PN 200 110 [data not shown]. Binding of [^{3}H]PN 200 110, measured to the same protein preparations employed for Bay K 8644 binding, was of significantly higher affinity, K_D = 1.4 ± 0.2 x 10^{-10}M, but with the same binding capacity B_{max} = 486 ± 44 fmoles/mg protein [n = 7]. The Hill slopes for Bay K 8644 and PN 200 110 binding, 1.02 and 0.94 respectively, were not significantly different from unity. The binding densities to this cardiac preparation are not significantly different to those reported previously for [^{3}H]nitrendipine, [^{3}H]nimodipine and [^{3}H]PN 200 110 of 420, 450 and 470 fmoles/mg protein respectively [14].

These data indicate that (-) S [^{3}H]Bay K 8644 defines binding sites in guinea pig cardiac membranes of lower affinity, but with the same capacity as those defined by the 1,4-dihydropyridine antagonist [^{3}H]PN 200 110. This suggests that these two ligands define the same population of binding sites in this cardiac preparation, consistent with earlier conclusions from pharmacologic [11,12] and radioligand binding [12,18] studies of competitive interactions between Bay K 8644 and 1,4-dihydropyridine antagonists. Additional support is provided by the observation that the S and R enantiomers of Bay K 8644 displace (-) [^{3}H]Bay K 8644 to an

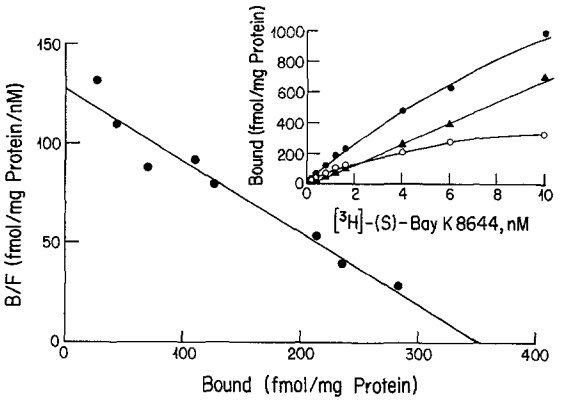

Figure 1. Scatchard representation of (-) S [^{3}H]Bay K 8644 specific binding to a microsomal membrane preparation of rat ventricle. Inset: saturation curves for (-) S [^{3}H]Bay K 8644 binding to microsomal membranes from rat ventricle. ●, Total binding, ▲ , nonspecific binding, ○, specific binding. Binding conditions were as indicated in Methods. Depicted is one representative plot. The experiment was replicated 7 times and the data are summarized in Table 1 together with the companion data for (+) [^{3}H]PN 200 110 binding to the same preparations.

TABLE 1. Binding Constants for (-) S [^{3}H]Bay K 8644 and (+) [^{3}H]PN 200 110 in Cardiac Preparations

Preparation	(-) S [^{3}H]Bay K 8644				(+) [^{3}H]PN 200 110			
	K_D x 10^{-8}M	B_{max} f moles/mg	n_H	n	K_D x 10^{-10}M	B_{max} f moles/mg	n_H	n
Guinea Pig ventricle membranes	0.29±0.04	337±69	1.02±0.05	7	1.47±0.28	485±64	0.98±0.07	
Rat neonatal ventricular myocytes								
5 mM K^+	5.15±0.14	63.1±5.8	1.05±0.05	8	35.3±7.8	50.1±11.0	0.99±0.02	
50 mM K^+	5.56±0.17	62.3±13.0	1.07±0.44	7	0.63±0.04	47.2±3.4	1.05±0.05	

equal extent with pseudoHill coefficients of 1.14 ± 0.14 and 0.90 ± 0.15 [n = 6-9] respectively [Table 2]. The binding constants are similar to those reported previously [12] for the displacement of [^{3}H]nitrendipine in cardiac and vascular smooth muscle preparations [Table 2]. However, the apparently greater affinity of S Bay K 8644 against [^{3}H]nitrendipine binding than against [^{3}H]Bay K 8644 binding suggests that even in membrane fragments differential stabilization of different channel states by antagonists and activators may occur.

1,4-Dihydropyridine interactions with Ca^{2+} channels are voltage-dependent [1,6,7,13] and measurements in membrane fragments may refer only or dominantly to the inactivated state of the channel. (-) [^{3}H]Bay K 8644 binding to cultured neonatal rat ventricular myocytes was measured under polarized [5 mM KCl] and depolarized [50 mM KCl] conditions to parallel measurements previously made with [^{3}H]PN 200 110 [16]. Specific binding, defined by 10^{-5}M S Bay K 8644 was 25 to 35% of the total binding over the concentration range 10^{-9} to 7 x 10^{-8}M. Measured at 37°C, binding was not significantly different under polarized or depolarized conditions with K_D values of 5 x 10^{-8}M and B_{max} values of 59-66 fmoles/mg protein [Figure 2; Table 1]. The binding capacity for Bay K 8644 is not significantly different from that defined by the antagonist PN 200 110.

That activator and antagonist 1,4-dihydropyridines define identical numbers of binding sites in both membrane and functional cell preparations suggests, consistent with much pharmacologic and radioligand binding competition data,

TABLE 2. Competition of Bay K 8644 Enantiomers for (-) S [^{3}H]Bay K 8644 and (±) [^{3}H]Nitrendipine Binding to Membrane Preparations of Smooth and Cardiac Muscle

Competing Ligand	(-) S [^{3}H]Bay K 8644			(+) [^{3}H] Nitrendipine[a]			
	G.P. ventricle			G.P. ventricle		Rat tail artery	
	K_I	n_H	n	K_I	n_H	K_I	n_H
(-) S Bay K 8644	1.66x10^{-8}	1.14±0.14	6	1.9x10^{-9}	0.94	1.2x10^{-9}	0.79
(+) R Bay K 8644	1.70x10^{-8}	0.90±0.15	9	8.6x10^{-9}	0.88	7.2x10^{-9}	0.76

[a] Data from Wei et al (12).

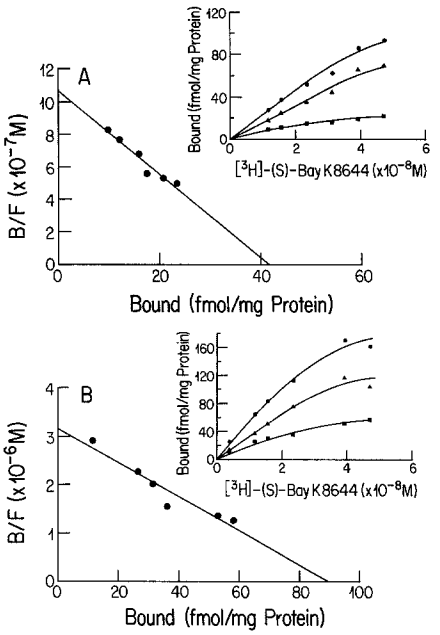

Figure 2. Scatchard representations of (-) [^{3}H]Bay K 8644 specific binding to polarized [A] and depolarized [B] rat neonatal rat myocytes. Inset: saturation curves for (-) [3H]Bay K 8644 binding. ●, total binding, ▲ , nonspecific binding, ■ , specific binding. Binding conditions were as indicated in Methods. Depicted is one representative plot. The experiments were replicated 7-8 times and the data are summarized in Table 1.

that a single 1,4-dihydropyridine binding site accomodates both ligand types and that the consequences of this interaction are both ligand and voltage sensitive. However, Kokubun et al [19], using a similar preparation of cultured cardiac cells to that described here, observed that under polarized conditions an activator 1,4-dihydropyridine potentiated the binding of the antagonist (+) [^{3}H]PN 200 110. This was interpreted as support for the existence of separate, but interacting, sites for activator and antagonist 1,4-dihydropyridines postulated previously [20]. The present work did not, however, reveal any potentiation of [^{3}H]Bay K 8644 binding in the cardiac cell preparation under polarized or depolarized conditions by either enantiomer of Bay K 8644. Further studies are needed to resolve this issue of multiple binding sites for 1,4-dihydropyridines.

That the binding affinity of (-) [^{3}H]Bay K 8644 in cardiac cells is independent of membrane potential is in marked contrast to both electrophysiologic and radioligand binding data for 1,4-dihydropyridine antagonists where marked increases in affinity with depolarization are observed consistent with preferential interactions with an inactivated state of the Ca^{2+} channel [1,6,7,16]. However, the independence of affinity from membrane potential of S Bay K 8644 reported here is consistent with electrophysiologic data showing a transition from activator to antagonist properties with decreasing membrane potential in cardiac cells [13]. Such state independence likely also underlies the close

agreement between binding and pharmacologic affinities observed in cardiac muscle for a series of 1,4-dihydropyridine activators [21], in marked contrast to the situation with the corresponding antagonists. It is anticipated that there should exist a series of 1,4-dihydropyridines ranging in properties from activator to antagonist and characterized by different voltage dependencies according to the extent of selective interaction with the several available channel states.

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