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BRIEF COMMUNICATION

Buffer Effects on High Affinity [^{3}H]-Prazosin Binding in Brain and Spinal Cord

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HERR, D. W. AND R. B. MAILMAN. *Buffer effects on high affinity [^{3}H]-prazosin binding in brain and spinal cord.* PHARMACOL BIOCHEM BEHAV **32**(3) 831–834, 1989.—[^{3}H]-Prazosin binding was characterized in cortical and spinal membranes from Fischer 344N and Sprague-Dawley rats. Estimates of B_{max} and K_d values were comparable with earlier studies of these regions in the central nervous system (CNS). However, the K_d obtained using Tris buffer system was greater than when HEPES or phosphate buffer was used. These data indicate that high affinity [^{3}H]-prazosin binding in the homogenates of tissue from the CNS is affected critically by buffer selection.

Buffer Prazosin Alpha-adrenoreceptor Radioligand binding Cerebral cortex Spinal cord

RECEPTOR binding techniques allow the quantification and pharmacological characterization of specific receptor subtypes. Prazosin is an adrenergic antagonist which has been reported to have selectivity for α_1 receptors both in the periphery (6,13) and central nervous system (CNS) (9,15). Prior to undertaking a study of toxicant-induced effects on [^{3}H]-prazosin binding sites using a high specific activity (82 Ci/mmol) ligand, we examined the characteristics of binding of [^{3}H]-prazosin to recognition sites in the cerebral cortex and spinal cord of two strains of rats, in several common buffers. We now report the unexpected finding that the buffer used has significant influence on the apparent characteristics of this ligand recognition site, and might influence results or introduce experimental artifacts.

EXPERIMENTAL PROCEDURES

Animals

Male Fischer 344N rats (Charles River Laboratories, Raleigh, NC), approximately 9–13 weeks old, were housed in plastic home cages with corncob bedding in groups of four at the National Institute of Environmental Health Sciences. Food (NIH diet No. 31) and water were available at all times. The animal colony was maintained at a temperature of 21 ± 2°C and 50 ± 10% humidity, with a 12 hr light-dark cycle (lights on 0700–1900). Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), about 9–12 weeks of age, were housed in hanging wire cages in groups of four at the Biological Sciences Research Center at the University of North Carolina. Food (Rodent Laboratory Chow No. 5001, Purina Mills, Inc., St. Louis, MO) and water were continuously available. The animal facilities were maintained at 22 ± 2°C and 45 ± 5% humidity, with a 12 hr light-dark cycle (lights on 0700–1900).

Membrane Preparation

Animals were decapitated, the brains removed, placed on ice, and the frontal cerebral cortex (anterior to the optic chiasm) removed. The spinal cord was removed hydraulically, dissected free from spinal roots, and a 2.5–3 cm portion of the lumbar region taken for assay. Tissue was frozen on dry ice and stored at −70°C until binding was performed. The mean weights (±SEM) of cortex and spinal cord were 386 ± 12 and 263 ± 7 mg, respectively. These regions of the CNS were selected because [^{3}H]-prazosin binding had previously been characterized in these tissues (1, 2, 18). Tissue from three rats was pooled in each of the assays. Tissue was homogenized on ice using a PCU-2 Polytron (setting 4) for 10 sec in 30 ml of a 50 mM buffer of either HEPES (N-2-

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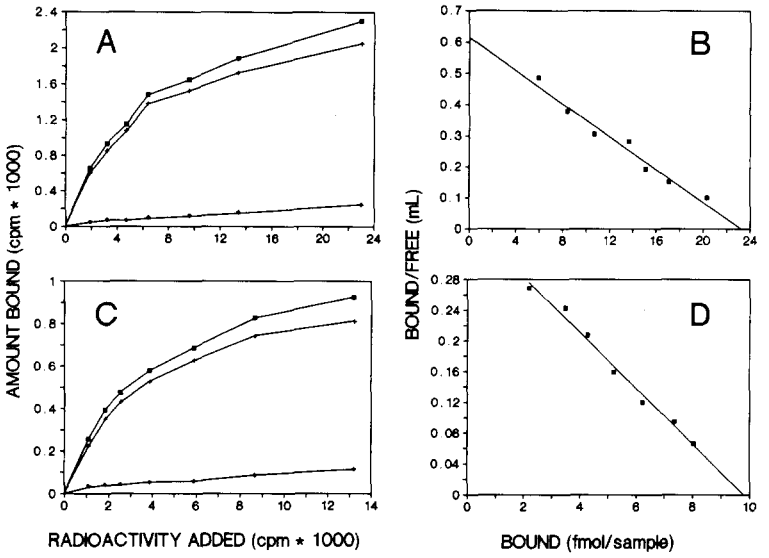

FIG. 1. (A) Saturation isotherm of [^{3}H]-prazosin binding in cortical tissue in HEPES buffer from Fischer 344N rats. See text for details. (■ = total bound; + = specific binding; △ = nonspecific binding). (B) Scatchard plot of specific binding in cortex in HEPES buffer from Fischer 344N rats. (C) Saturation isotherm of [^{3}H]-prazosin binding in spinal tissue in HEPES buffer from Fischer 344N rats. See text for details. (■ = total bound; + = specific binding; △ = nonspecific binding). (D) Scatchard plot of specific binding in spinal cord in HEPES buffer from Fischer 344N rats.

hydroxyethylpiperazine-N-2-ethanesulfonic acid, Research Organics, Inc., Cleveland, OH), Tris-HCl [(tris-hydroxymethyl) aminomethane, Sigma Chemical Co., St. Louis, MO], or K_2HPO_4-NaH_2PO_4 (pH 7.4 at 25°C). An aliquot of this homogenate was diluted to 20 ml with buffer, and centrifuged at 40,000 × g (cortex), or 32,570 × g (spinal cord), for 10 min at 4°C. After discarding the supernatant, the pellet was resuspended in 20 ml cold buffer and centrifuged as before; this washing procedure was performed twice. The final pellet was resuspended to an original wet tissue weight of 3 mg/ml to obtain final concentrations of ca. 0.12–0.18 mg protein/ml.

Binding Assay

In order of their addition, 100 μl of 7-methoxy-[^{3}H]-prazosin (82 Ci/mmol; Dupont/New England Nuclear, Boston MA), 100 μl of either buffer, WB4101 [2 - (2,6 -dimethoxyphenoxyethyl)aminomethyl - 1,4 - benzodioxane hydrochloride, Research Biochemicals Inc., Natick, MA], or phentolamine (Ciba-Geigy Corp., Summit, NJ); and 800 μl of tissue homogenate were added to culture tubes (12 × 75 mm). Tissue homogenate and tubes were maintained separately on ice until mixing. Nonspecific binding was defined by 10 μM phentolamine (cortex) or 1 μM WB4101 (spinal cord) (1, 2, 14, 17, 18). [^{3}H]-Prazosin concentrations were varied over the ranges of 0.02–1.024 nM (cortex) and 0.012–0.169 nM (spinal cord). Triplicate samples were vortexed and incubated with shaking for 30 min at 25°C. Binding was terminated by vacuum filtration over 1.5 μm filters using a Skatron Cell Harvester (Skatron Inc., Sterling, VA). Filters were then rapidly rinsed with 15 ml ice-cold buffer and allowed to air dry. Radioactivity was quantified by liquid scintillation spectrometry after adding 3.2 ml of Scintiverse E (Fisher Scientific Co., Fair Lawn, NJ). An LKB Wallac counter (Model 1219 Rackbeta, LKB Instruments, Inc., Gaithersburg, MD) with a counting efficiency of 56% was used.

In three separate experiments, a single cortex and corresponding spinal cord from Fischer 344N rats were individually homogenized in 20 ml HEPES buffer (pH = 7.4 at 25°C). Tissue homogenates were centrifuged (32,750 × g) for 10 min at 4°C and washed twice as previously described. The final pellets were resuspended to an original tissue weight of 3–5 mg/ml, resulting in final concentrations of ca. 0.11–0.32 mg protein/ml. The binding assay was performed essentially as previously described. Prazosin concentrations were varied over the range of 0.01–1.5 nM, with nonspecific binding defined by 1 μM WB4101 in both cortical and spinal membranes. Protein concentration was estimated using bovine serum albumin as a standard (12).

Data Analaysis

Each point was assayed in triplicate. Results were expressed as average binding on a per mg protein basis. K_d, B_{max}, and n_H values were obtained using linear regression of Scatchard or Hill plots on Lotus 1,2,3 templates. No significant differences were seen when the data were analyzed using McPherson's EBDA-LIGAND.

RESULTS

The binding of [^{3}H]-prazosin to cortical and spinal tissue was saturable and of high affinity (Fig. 1). Linear Scatchard plots (linear correlation coefficients >0.98) were consistent with single populations of sites for [^{3}H]-prazosin in cortical and spinal tissues ($n_H = 1.05 \pm 0.05$). The characteristics of this recognition site were similar when a comparison was made between tissue from Fischer 344N and Sprague-Dawley rats (Table 1).

Several effects of buffer should be noted. The apparent dissociation constant (K_d) found in experiments using Tris-HCl buffer was consistently 1.6–3.5 fold larger (i.e., binding was of lower affinity) than estimates when HEPES or K_2HPO_4-NaH_2PO_4 buffers were employed (Table 1). Conversely, although the

TABLE 1

ESTIMATES OF K_d AND B_{max} VALUES FOR [^{3}H]-PRAZOSIN BINDING IN CORTICAL AND SPINAL MEMBRANES FROM FISCHER 344N AND SPRAGUE-DAWLEY RATS[a]

	Fischer 344N		Sprague-Dawley	
	K_d*	B_{max}*	K_d	B_{max}
		Cortex		
Tris-HCl	0.07	195.0	0.07	215.1
HEPES	0.04	139.2	0.03	150.2
Na-KPO_4	0.02	157.3	0.02	180.1
		SpinalCord		
Tris-HCl	0.08	77.6	0.07	71.4
HEPES	0.03	53.3	0.02	52.2
Na-KPO_4	0.05	66.9	0.04	62.3

[a]Data are averages of triplicate points using pooled tissues from three rats.

*Units for K_d are nM. Units for B_{max} are fmol/mg protein.

calculated maximal number of binding sites appeared to be greater in Tris-HCl buffer than in HEPES or phosphate buffer, the variance (standard error = 16–18% of mean) associated with the B_{max} estimates from single tissue experiments suggested that there may not be real differences in the B_{max} values. The estimated K_d and B_{max} values ($\pm$SEM) in single tissue experiments were as follows: 1) cortex -0.02 ± 0.006 nM and 193 ± 35 fmol/mg protein; and 2) spinal cord -0.04 ± 0.01 nM and 71 ± 12 fmol/mg protein. In these studies, the Hill coefficient did not change, and was not significantly different from 1.0.

In an additional experiment, when Fischer 344N cortical membranes were homogenized and washed in HEPES with the final incubation occurring in Tris-HCl, there was a 55.8% reduction in the binding of a single concentration (0.03 nM) of [^{3}H]-prazosin when compared to membranes prepared completely in HEPES buffer (data not shown). In all experiments, the pH of the buffer was determined, and fell within ± 0.05 units of 7.4 at 25°C.

DISCUSSION

The results of these experiments indicate that [^{3}H]-prazosin binds to a single population of sites on cortical and spinal membrane preparations, and that the binding is both saturable and of high affinity. Our B_{max} estimates are in agreement with previous reports utilizing [^{3}H]-prazosin binding in the same regions of the CNS (1, 2, 9, 10, 14, 18). Our K_d estimates are lower than those reported by these authors (*vide supra*), but are similar to values recently found in cortical tissue and in the periphery (3–5, 11, 16, 19). An interesting observation in this series of experiments is that the estimated K_d values for [^{3}H]-prazosin binding were 1.6–3.5 fold higher in Tris-HCl buffer than in HEPES or phosphate buffers. Because a similar pattern of differences in K_d values were seen in both Fischer 344N and Sprague-Dawley rats, the present studies indicate that these results are not due to the use of a particular strain of rat. A similar effect of Tris-HCl buffer on the affinity of [^{3}H]-prazosin binding has been noted for peripheral tissues (11), and casually mentioned for cortical tissues (21).

Buffer composition may often have substantial effects on ligand-receptor interactions. In some cases, this can be explained by chelation of trace metals (7,8), or by direct effects on recognition sites (20). However, the effect shown in the present work may not be explicable on the basis of either of these mechanisms. Tris has also been reported to have effects on membrane-bound functional processes by undetermined mechanisms (22); this may be related to the present findings. In any event, differences of the magnitude we have shown often would be considered to be of biological significance. Thus, buffer composition may be a critical experimental variable when this ligand is used for either homogenate or autoradiographic studies.

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