Biochemical Pharmacology, Vol. 31, No. 4, pp. 589–590, 1982.
Printed in Great Britain.

0006-2952/82/040589-02 $03.00/0

SHORT COMMUNICATIONS

[^{3}H]Diazepam binding sites on rat heart and kidney

(*Received* 14 *March* 1981; *accepted* 3 *August* 1981)

The CNS benzodiazepine receptors have been investigated intensely since their discovery in 1977 [1–3]. Less well known, and largely ignored, are the related but pharmacologically distinguishable peripheral benzodiazepine binding sites, which initially were found in kidney, liver and lung [4]. Recently, we reported the detailed characterization of these sites in rat mast cells [5], platelets [6], and guinea pig ileum longitudinal muscle*. Brief reports of these sites in kidney [7, 8] and heart [9] have also appeared. To facilitate their further studies, we present here the basic binding parameters of the peripheral sites in rat heart and kidney. Crude membrane fractions of heart, kidney and brain from male Wistar rats (Charles River, Boston, MA) were prepared as described previously [9], and [^{3}H]diazepam binding assay was performed according to Taniguchi *et al.* [5]. In the routine assay, 0.5 mg protein and 3.6 nM [^{3}H]diazepam (76.8 Ci/mmole, New England Nuclear Corp., Boston, MA) were incubated in 50 mM sodium phosphate buffer (pH 7.4) for 15 min at 0°. All assays were done in duplicate.

When the crude membrane fractions of heart and kidney were incubated with increasing concentrations of [^{3}H]diazepam (1.4 to 109.0 nM), specific binding in both tissues reached saturation (Fig. 1), while nonspecific binding increased linearly (data not shown). Scatchard analysis resulted in one straight line, suggesting a single class of non-cooperative binding sites. The K_D in the heart was 34 ± 5 nM and the B_{max} was 967 ± 108 fmoles/mg protein; the corresponding values in the kidney were 35 ± 4 nM and 1932 ± 86 fmoles/mg protein respectively.

The binding of [^{3}H]diazepam reached equilibrium after 7 min in both heart and kidney preparations (Fig. 2). The bimolecular association rate constants (K_{+1}) were calculated to be 2.13 ± 1.08 × 10^7 M^{-1} min^{-1} in the heart and 1.38 ± 0.80 ± 10^7 M^{-1} min^{-1} (mean ± S.E., N = 3) in the kidney. Dissociation of [^{3}H]diazepam binding was first order and rapid, with $T_{1/2}$ = 1.11 ± 0.06 min (N ± 3) in the heart and 1.17 ± 0.09 min (N = 3) in the kidney (data not shown). The dissociation rate constants were 0.61 ± 0.05 min and 0.60 ± 0.05 min for heart and kidney respectively. K_D values derived from the rate constants were 52 ± 24 nM for the heart and 72 ± 24 nM for the kidney. These are in reasonable agreement with the estimates from Scatchard analysis. When the dissociation experiment was carried out at 37°, we could not measure the rate of dissociation accurately because of the extremely rapid time course, the $T_{1/2}$ being less than 5 sec in both heart and kidney (data not shown).

The binding of [^{3}H]diazepam to heart and kidney membranes was specific, since it was not affected by a 10 μM concentration of each of the following: norepinephrine, epinephrine, phentolamine, propranolol, acetylcholine, atropine, carbachol, serotonin, histamine, diphenhydramine, hexamethonium, morphine, levallorphan and γ-aminobutyric acid (GABA). Ro5-4864, a ligand selective for the peripheral benzodiazepine binding sites, was five times more potent than diazepam in displacing [^{3}H]diazepam binding in both tissues (Table 1). Clonazepam, a specific ligand for the central benzodiazepine sites, was ineffective in displacing [^{3}H]diazepam binding. Hence, these sites could be characterized as the peripheral type. Specific binding of [^{3}H]diazepam increased linearly with increasing protein concentrations in the range 0.1 to 0.6 mg from either the heart of kidney membranes (data not shown).

The benzodiazepine binding sites in heart and kidney, in common with those in mast cells and platelets, are specific, saturable, GABA independent, and of the peripheral type, based on the relative potencies of Ro5-4864 and clonazepam in inhibiting [^{3}H]diazepam binding. The binding of [^{3}H]diazepam to heart and kidney was also maximal at 0° (data not shown). However, the decrease in binding at higher temperatures was at least partly due to the fast off rate, since during the filtration assay the membrane was exposed to wash medium for about 5 sec, which should be sufficient to wash off more than half the binding. The temperature dependency of the peripheral [^{3}H]diazepam binding, therefore, may be an artifact of the fast dissociation rate when using the filtration assay. Studies utilizing other techniques such as separation of bound and free ligand by centrifugation are needed to ascertain whether the peripheral benzodiazepine binding is temperature dependent.

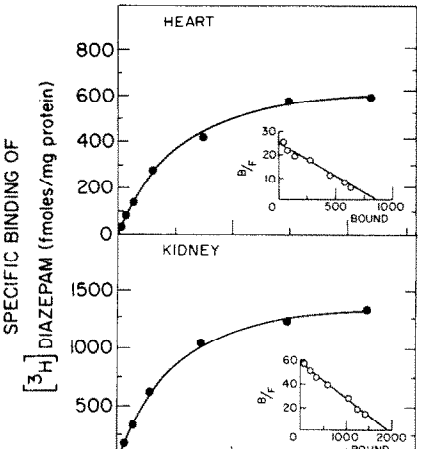

Fig. 1. Typical experiments on saturation of specific [^{3}H]diazepam binding to Wistar rat heart or kidney homogenates as a function of increasing concentrations of [^{3}H]diazepam (1.4 to 109 nM) for 15 min at 0°, as described in the text. The inset shows a Scatchard plot of specific [^{3}H]diazepam binding. Bound = fmoles of specifically bound [^{3}H]diazepam per mg protein; free = concentration of [^{3}H]diazepam present in the incubation medium. The regression lines (r = 0.99) indicate a K_D of 33.3 nM for the heart and 33.3 nM for the kidney, while B_{max} is 850 fmoles/mg protein for the former and 1968 fmoles/mg protein for the latter.

* Unpublished data.

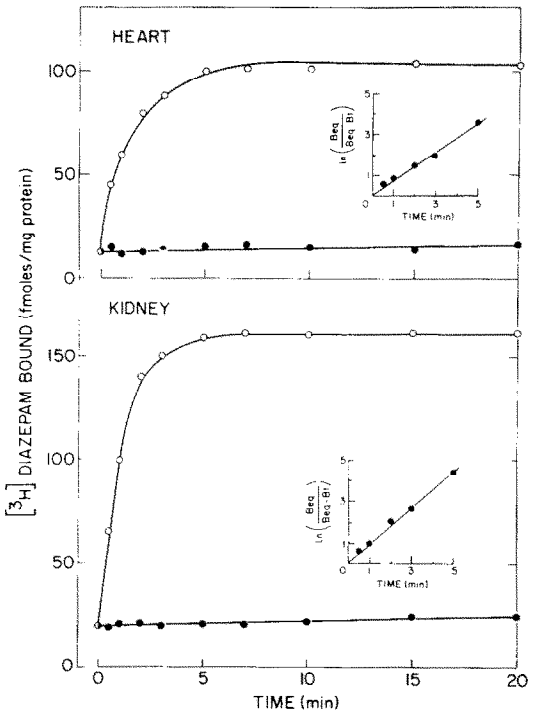

Fig. 2. Typical experiments on the time course of [^{3}H]diazepam binding to heart or kidney homogenates. The homogenates were incubated as described in the text for various periods of time. Open circles represent specific binding; closed circles represent nonspecific binding. The inset plots a regression line that was determined by least-squares fit. B_{eq} = fmoles of specifically bound [^{3}H]diazepam at equilibrium, and B_t = fmoles of specifically bound [^{3}H]diazepam at time t. K_{obs} is the slope of this line and is 0.75 for the heart and 0.80 for the kidney. K_{-1} can be calculated from the equation $K_{-1} = (K_{obs} - K_{+1})/$ [^{3}H]diazepam, where K_{+1} is the rate constant of dissociation and [^{3}H]diazepam is the concentration of the labelled ligand (3.6 nM).

While it is evident that the peripheral benzodiazepine binding sites exist in heart and kidney, their functional role or physiological significance remains unknown. There are reports of the effects of diazepam on various functions of the mammalian heart. Some investigators claim that diazepam has anti-arrhythmic properties in humans [10, 11] but this is contradicted by others [12, 13]. Abel *et al.* [14] found that diazepam increased cardiac contractility secondarily to an increase in coronary blood flow. Daniell [15], however, reported that diazepam increased coronary blood flow and cardiac output but decreased blood pressure, heart rate and myocardial contractile force. Whether any of these disparate findings is related to the benzodiazepine binding sites in the heart is an open question.

As for the binding sites in the kidney, Regan *et al.* [8] reported that the B_{max} of the benzodiazepine binding sites is higher in deoxycorticosterone/salt, uninephrectomized, hypertensive rats compared to controls. We, on the other hand, found that the renal binding site number is decreased in spontaneously hypertensive rats compared to WKY controls at 4 and 20 weeks of age [9]. These data suggest that in animal models of hypertension the number of benzodiazepine binding sites can be altered, although the relationship of these binding sites to the disease is questionable.

Table 1. Displacement by three benzodiazepines for [^{3}H]diazepam binding to different tissues*

Tissue	K_i (nM)		
	Diazepam	Clonazepam	Ro5-4864
Heart	43 ± 5	> 10,000	8 ± 2
Kidney	49 ± 10	> 10,000	10 ± 1
Brain	5.3 ± 0.6	2.9 ± 0.3	> 10,000

* Binding was assayed as described in the text. Each value is the mean ± S.E. of five experiments. A value for the inhibition constants, K_i, has been calculated from the equation, $K_i = K_D \cdot IC_{50}/(L + K_D)$, where L = concentration of [^{3}H]diazepam (3.6 nM).

The effects of benzodiazepines on heart and kidney functions are still unclear and in need of extensive investigation. The presence of benzodiazepine binding sites in these organs provides an additional tool for studies to clarify what the effects are and may lead to a better understanding of the underlying physiology.

Department of Physiological Chemistry and Pharmacology
Roche Institute of Molecular Biology
Nutley, NJ 07110, U.S.A.

TAKASHI TANIGUCHI*
JAMES K. T. WANG†
SYDNEY SPECTOR‡

REFERENCES

1. R. F. Squires and C. Braestrup, *Nature, Lond.* **266**, 732 (1977).
2. H. Möhler and T. Okada, *Science* **198**, 849 (1977).
3. C. R. Mackerer, R. L. Kochman, B. A. Bierschenk and S. S. Bremner, *J. Pharmac. exp. Ther.* **206**, 405 (1978).
4. C. Braestrup and R. F. Squires, *Proc. natn. Acad. Sci. U.S.A.* **74**, 3805 (1977).
5. T. Taniguchi, J. K. T. Wang and S. Spector, *Life Sci.* **27**, 171 (1980).
6. J. K. T. Wang, T. Taniguchi and S. Spector, *Life Sci.* **27**, 1881 (1980).
7. P. J. Syapin and P. Skolnick, *J. Neurochem.* **32**, 1047 (1979).
8. J. W. Regan, H. I. Yamamura, S. Yamada and W. R. Roeske, *Eur. J. Pharmac.* **67**, 167 (1980).
9. T. Taniguchi, J. K. J. Wang and S. Spector, *Eur. J. Pharmac.* **70**, 587 (1981).
10. G. R. Van Loon, *Can. med. Assoc. J.* **215**, 1288 (1968).
11. C. Papp, *Br. Heart J.* **31**, 267 (1969).
12. Muenster, M. S. Rosenberg, R. A. Carleton and J. S. Graettinger, *J. Am. med. Ass.* **199**, 758 (1967).
13. F. H. N. Spracklen, R. J. Chambers and V. Schrire, *Br. Heart J.* **32**, 827 (1970).
14. R. M. Abel, R. L. Reis and R. N. Staroscik, *Br. J. Pharmac.* **38**, 620 (1970).
15. H. B. Daniell, *Eur. J. Pharmac.* **32**, 58 (1975).

* Present address: Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan.
† Department of Pharmacology, Columbia University, New York, NY 10032, U.S.A.
‡ Author to whom all correspondence should be addressed.