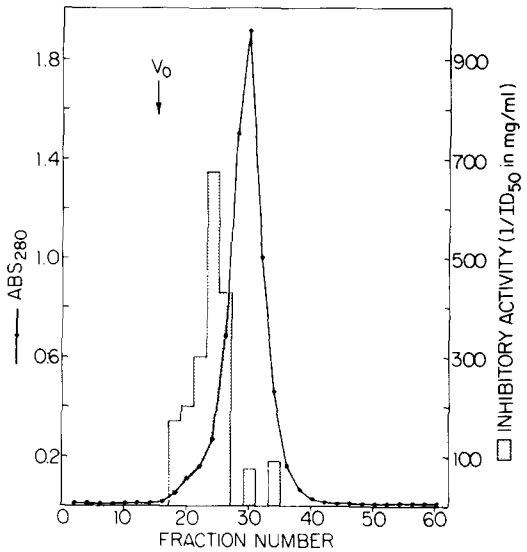

Fig. 1. Sephadex G-25 elution profile of compound 48/80. A 20-mg sample of compound 48/80 (Sigma Lot 20F-0396) was applied, and fractions of 2 ml were collected. The fractions were lyophilized and redissolved in 20 mM acetic acid for biological assay. The bars represent the relative inhibitory activities, $1/IC_{50}$ expressed in mg/ml, calculated from concentration curves obtained in the MLC assay.

pound 48/80 and somatostatin, a peptide hormone, while differing greatly in cationic charge, have similar molecular shapes and mast cell degranulating activity [17]. The concept is supported by calculations of the IC_{50} based on concentration of amine groups (used here as a measure of relative cationic nature) which indicate that 12,000 molecular weight poly-L-lysine requires about 120 μM amine. The most active G-25 fraction of compound 48/80 requires an estimated 6–10 μM amine, or less than one-tenth that of 12,000 molecular weight poly-L-lysine. Thus, it would seem that the growth inhibition exhibited by the low molecular weight polycations is not simply due to binding up of cell surface negative charges. These polycations may have a shape similar enough to polypeptides such that they can fit into receptor sites, inhibit enzymes such as transglutaminase, or cause other membrane or enzyme perturbations [3]. Further experiments will be necessary to elaborate the exact mechanism and other variables of this inhibition.

In summary, compound 48/80 and other low molecular weight polycations have been found to be potent inhibitors of normal and leukemic lymphocyte proliferation. On a molar basis these polycations were as active as poly-L-lysine or hexadimethrine, polycations many times larger. These results suggest that certain low molecular weight polycations have a molecular shape/size which makes them more potent inhibitors of proliferation than their degree of cationicity would indicate. Such low molecular weight polycations may provide a route to new antimitotic or immunosuppressive drugs.

Acknowledgement—We thank Ms. Janelle Taylor-Norton for excellent technical assistance.

Virginia Mason Research Center
Seattle, WA 98101, U.S.A.

LEONARD M. PATT*
JOHN C. HOUCK

* Address all correspondence to: Dr. Leonard M. Patt, Virginia Mason Research Center, 1000 Seneca St., Seattle, WA 98101, U.S.A.

REFERENCES

1. E. Mayhew, J. P. Harlos and R. L. Juliano, *J. memb. Biol.* **14**, 213 (1973).
2. S. E. Kornguth and M. A. Stahmann, *Cancer Res.* **21**, 907 (1961).
3. T. C. Theoharides, *Life Sci.* **27**, 703 (1980).
4. L. J. Arnold, A. Dagan, S. D. Simon and N. O. Kaplan, *Proc. Am. Ass. Cancer Res.* **19**, 114 (1978).
5. H. J. P. Ryser and W. C. Shen, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3867 (1978).
6. W. C. Shen and H. J. P. Ryser, *Proc. natn. Acad. Sci. U.S.A.* **78**, 7589 (1981).
7. A. Novogradsky, *Nature, Lond.* **250**, 788 (1974).
8. B. Larsen and I. Heron, *Experientia* **34**, 1224 (1978).
9. L. J. Arnold, A. Dagan, J. Gutheil and N. O. Kaplan, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2346 (1979).
10. R. Baltzly, J. S. Buck, E. J. DeBeer and F. J. Webb, *J. Am. chem. Soc.* **71**, 1301 (1949).
11. E. F. Kiefer and J. F. Weber, *J. med. Chem.* **16**, 1292 (1973).
12. W. D. M. Paton, *Br. J. Pharmac.* **6**, 499 (1951).
13. G. W. Read and J. F. Lenney, *J. med. Chem.* **15**, 320 (1972).
14. M. J. Ortner, R. H. Sik, C. F. Chignell and E. A. Sokoloski, *Molec. Pharmac.* **15**, 179 (1979).
15. L. M. Patt, J. M. Gleisner, D. M. Barrantes and J. C. Houck, *Pharmacology* **23**, 117 (1981).
16. L. M. Patt, D. M. Barrantes and J. C. Houck, *Biochem. Pharmac.* **31**, 2353 (1982).
17. T. C. Theoharides and W. W. Douglas, *Eur. J. Pharmac.* **73**, 131 (1981).

Biochemical Pharmacology, Vol. 32, No. 3, pp. 567–570, 1983.
Printed in Great Britain.

0006-2952/83/030567-04 $03.00/0

Further evidence that vascular serotonin receptors are of the $5HT_2$ type

(*Received* 27 *March* 1982; *accepted* 5 *August* 1982)

Serotonergic receptor mechanisms in vascular tissue have been studied extensively, but their characterization in relation to brain serotonin receptors has been attempted only recently. In brain, two subtypes of serotonin receptors

have been defined, one radiolabeled by [^{3}H]serotonin, which has been termed a $5HT_1$ receptor, and a second serotonergic receptor radiolabeled by [^{3}H]spiperone which has been termed a $5HT_2$ receptor [1]. Based on this distinction, we [2] and others [3, 4] have recently presented evidence that serotonergic receptors mediating contraction in blood vessels are of the $5HT_2$ type. Ketanserin, an antagonist with high affinity and selectivity for $5HT_2$ binding sites in brain [5], is a new antihypertensive agent suggested to act by blocking vascular $5HT_2$ receptors [6].

In this report, using a series of eight compounds that interact with serotonergic receptors in both the brain and vascular tissue, we have obtained further evidence to support the idea that vascular serotonergic receptors mediating contraction are $5HT_2$ receptors. Furthermore, we sought to determine whether blood vessels contained serotonergic receptors other than those mediating contraction. Since contractile responses to serotonin appear to be mediated by interaction with $5HT_2$ receptors, we determined if blockade of these receptors with antagonists such as spiperone might unmask a vasodilatory response which could be mediated by the interaction of serotonin with $5HT_1$ receptors. However, we were unable to demonstrate any additional effect of serotonin on smooth muscle that might be mediated by receptors other than $5HT_2$ in vascular tissue.

Methods

Isolation of vascular tissue. Male Wistar rats (150–300 g) (Harlan Industries, Inc., Cumberland, IN) were killed by a blow to the head. External jugular veins were dissected free of connective tissue, cannulated *in situ* with polyethylene tubing (PE-50, outside diameter = 0.97 mm) and placed in Petri dishes containing Krebs' bicarbonate buffer (see below). The tips of two 30-gauge stainless-steel hypodermic needles bent into an L-shape were slipped into the polyethylene tubing. Vessels were gently pushed from the cannula onto the needles. The needles were then separated so that the lower one was attached with thread to a stationary glass rod and the upper one was tied with thread to the transducer. This procedure for ring preparations (circular smooth muscle) of blood vessels has been described previously [7].

Tissues were mounted in organ baths containing 10 ml of modified Krebs' solution of the following composition (millimolar concentrations): NaCl, 118.2; KCl, 4.6; $CaCl_2 \cdot 2H_2O$, 1.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; dextrose, 10.0; and $NaHCO_3$, 24.8. Tissue bath solutions were maintained at 37° and aerated with 95% O_2–5% CO_2. An initial optimum resting force of 1 g was applied to the jugular veins [8]. Isometric contractions were recorded as changes in grams of force on a Beckman Dynograph with Statham UC-3 transducers and microscale accessory attachment. Tissues were allowed to equilibrate 1–2 hr before exposure to drugs.

Determination of apparent dissociation constants. After control responses to serotonin were obtained, vessels were incubated with appropriate concentrations of antagonist for 1 hr, a procedure recommended by Furchgott [9]. Responses to serotonin were then repeated in the presence of antagonist.

Apparent antagonist dissociation constants (K_B) were determined for each concentration of antagonist according to the following equation [10]:

$$K_B = \frac{[B]}{[\text{dose ratio} - 1]}$$

where $[B]$ is the concentration of the antagonist, and the dose ratio is the ED_{50} of the agonist in the presence of the antagonist divided by the control ED_{50}. These results were then expressed as the negative logarithm of the K_B (i.e. $-\log K_B$). Calculations were performed with the aid of a computer and digital plotter as previously described [11].

The data were also analyzed according to the procedure of Arunlakshana and Schild [12]. The dose ratio was determined at various concentrations of antagonist. According to Arunlakshana and Schild [12], if blockade is competitive, under equilibrium conditions, a plot of the logarithm of (dose ratio − 1) against the negative logarithm of the molar concentration of antagonist should yield a straight line whose slope is 1 and intercept along the abscissa is the pA_2 which is equal to $-\log K_B$.

Cortical binding to $5HT_2$ and $5HT_1$ receptors. Brain tissue was obtained from 150 to 200 g male Wistar rats. The cerebral cortex was dissected, homogenized, and prepared according to the method described by Nelson *et al.* [13] using a preincubation in buffer without added monoamine oxidase inhibitor in order to eliminate endogenous serotonin. For receptor binding, an amount of membrane preparation equivalent to 250–350 mg of protein was used per sample in 1 ml of Tris buffer. The assay for serotonin binding was done following the method of Bennett and Snyder [14] and that for spiperone binding according to Peroutka and Snyder [1]. Non-specific binding of [^{3}H]serotonin and [^{3}H]spiperone was determined in the presence of 10^{-5} M serotonin or 10^{-6} M lysergic acid diethylamide (LSD), respectively, and specific binding was calculated as the difference between total binding without added non-radioactive compound and the non-specific binding. The IC_{50} values were determined as the amount of substance causing 50% inhibition of the specific binding using ten different concentrations in the range of 10^{-9} to 10^{-4} M. The concentration of [^{3}H]serotonin (sp. act. 17.6 Ci/mmole, Amersham Corp., Arlington Heights, IL) in each sample was 2.3 to 2.6 nM and that of [^{3}H]spiperone (sp. act. 20 Ci/mmole, Amersham) was 0.5 to 0.7 nM.

Results

Eight compounds [spiperone, ketanserin, mianserin, trazodone, mepiprazole, benzoctamine, *m*-trifluoromethylphenylpiperazine (TFMPP) and *m*-chlorophenylpiperazine (CPP)] were evaluated for their abilities to antagonize serotonin-induced contractions in the rat jugular vein and to displace the binding of [^{3}H]spiperone ($5HT_2$ receptors) or [^{3}H]serotonin ($5HT_1$ receptors) in brain cortical membranes. An excellent correlation was found between affinity for vascular serotonin receptors and affinity for $5HT_2$ receptors in brain cortical membranes (Fig. 1). In contrast,

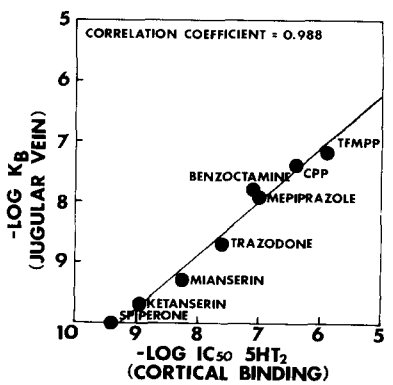

Fig. 1. Correlation of the negative logarithm of the dissociation constant (an estimate of affinity) of each antagonist for vascular serotonin receptors to the negative logarithm of the IC_{50} for binding (an estimate of affinity) of each antagonist to brain cortical receptors radiolabeled with [^{3}H]spiperone. A highly significant correlation coefficient of 0.988 was found. Each point represents the indicated serotonergic antagonist. *m*-Trifluoromethylphenylpiperazine and *m*-chlorophenylpiperazine are abbreviated as TFMPP and CPP respectively.

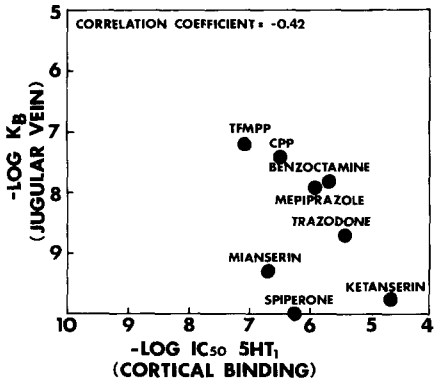

Fig. 2. Correlation of the negative logarithm of the dissociation constant (an estimate of affinity) of each antagonist for vascular serotonin receptors to the negative logarithm of the IC_{50} for binding (an estimate of affinity) of each antagonist to brain cortical receptors radiolabeled with [^{3}H]serotonin. The correlation coefficient was −0.42. Each point represents the indicated serotonergic antagonist. *m*-Trifluoromethylphenylpiperazine and *m*-chlorophenylpiperazine are abbreviated as TFMPP and CPP respectively.

no direct correlation was found between receptors mediating vascular contraction and affinity for $5HT_1$ receptors in brain cortical membranes (Fig. 2).

The next question addressed was whether any serotonergic responses other than those mediated by interaction with $5HT_2$ receptors could be demonstrated in vascular smooth muscle. We reasoned that, if vascular contraction were due to interaction with $5HT_2$ receptors, perhaps $5HT_1$ receptors might be present and might mediate relaxation to serotonin. To examine this possibility, rat jugular veins were incubated with a high concentration of spiperone (10^{-7} M). A moderate degree of tone was induced in the preparations with potassium chloride (20–30 mM), and serotonin was then added. In tissues not treated with spiperone, serotonin (1.8×10^{-7} M) caused a marked contraction (Fig. 3). In other tissues treated with spiperone, serotonin (5.4×10^{-6} M) neither relaxed nor contracted the jugular veins, whereas higher concentrations of serotonin (1.8×10^{-5} M) overcame the blockade by spiperone and caused a modest contraction (Fig. 3). No concentration-dependent relaxation to serotonin could be demonstrated in this vascular tissue.

Discussion

Recently, we [2] and Leysen *et al.* [3, 4] have proposed that serotonin receptors mediating contraction are of the $5HT_2$ type, as defined in the central nervous system by Peroutka and Snyder [1, 15]. In the present report, we have extended our evidence to a series of eight different compounds, all of which bind to the $5HT_2$ receptor in brain cortical tissue and antagonize vascular serotonin contractions. These two effects were highly correlated (correlation coefficient = 0.988), suggesting that those receptors responsible for contraction to serotonin in blood vessels are the same as the $5HT_2$ receptors radiolabeled by spiperone in the brain.

Since relatively high concentrations of serotonin are required to displace tritiated spiperone from its binding sites in the frontal cortex, the idea advanced by Peroutka and Snyder [1] that these binding sites represent serotonin receptors (designated the $5HT_2$ receptor subtype) has been challenged [16]. Ennis and Cox [17] recommended that, until there was pharmacological evidence for the existence of a receptor equivalent to the $5HT_2$ binding site, the validity of the claim that it represents a serotonin receptor should remain in doubt. Our data ([2]; this paper) and those of Leysen *et al.* [3, 4], indicating that the vascular serotonin receptor mediating contraction is equivalent in specificity to the $5HT_2$ binding site in brain, constitute pharmacologic evidence supporting the claim that this site represents a serotonin receptor. The vascular receptor mediating serotonin-induced contraction has now been demonstrated to be of the $5HT_2$ subtype in rat aorta [2], caudal artery [3, 4], and jugular vein (Fig. 1).

Peroutka *et al.* [18] reported that the relative potency of antagonists to inhibit 5-hydroxytryptophan-induced head twitches in mice correlated with their affinity for $5HT_2$ binding sites. Leysen *et al.* [4] also found that the order of potency among several compounds in antagonizing tryptamine-induced convulsions and mescaline-induced head twitches in rats correlated with their potency to block [^{3}H]ketanserin binding to brain membranes. Since [^{3}H]ketanserin is a selective ligand for the $5HT_2$ binding sites [4], their data add to the evidence linking receptor functions to the $5HT_2$ binding sites. In general, other

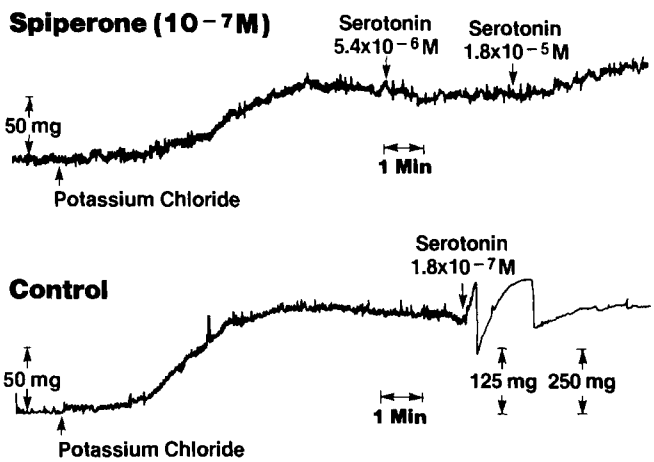

Fig. 3. Dynograph recordings of individual rat jugular veins. Tissues were contracted to a moderate degree of tone with potassium chloride. When contractile tone reached a steady state, serotonin was added at arrows. Top: tissue was incubated with spiperone (10^{-7} M). Bottom: control tissue.

attempts to correlate receptor-mediated functional changes with radioligand binding to serotonin receptors have not been successful. For instance, Ennis and Cox [17] studied central serotonin receptors influencing electrically stimulated or spontaneous overflow of radioactive serotonin or dopamine from raphe or striatal slices, respectively, and concluded that these receptors did not correlate with either serotonin or spiperone binding sites defined with radioligands. In addition, Langer and Moret [19] reported that the presynaptic autoreceptor modulating serotonin release from slices of rat hypothalamus differs pharmacologically from both the $5HT_1$ and $5HT_2$ subtypes of receptors. Rogawski and Aghajanian [20] studied serotonin receptors whose activation suppresses spontaneous firing of single dorsal raphe serotonergic neurons in rat brain and found that these did not have characteristics like the spiperone-binding or the serotonin-binding sites (the best correlation was with tritiated LSD binding). The affinity of antagonists for blocking quipazine-induced elevation of serum corticosterone in rats also did not correlate with their affinity for serotonin or spiperone binding sites (R. W. Fuller and N. R. Mason, data to be published).

Although Peroutka *et al.* [18] reported that the relative potency of antagonists inhibiting serotonin-stimulated adenylate cyclase correlated with their affinity for $5HT_1$ receptors, in general there appears to be more extensive data at this stage associating functions with $5HT_2$ binding sites than with $5HT_1$ binding sites. We found no effects of serotonin on vascular smooth muscle that might be attributed to $5HT_1$ receptors. Thus, using the rat jugular vein as our model system for evaluating serotonergic responses, only serotonin receptors analogous to $5HT_2$ could be demonstrated.

Currently, there are three lines of evidence to support the contention that vascular receptors responsible for contraction to serotonin are $5HT_2$ receptors. First, analysis of the receptor interactions of serotonin and quipazine in two vascular tissues was consistent with an interaction with $5HT_2$ but not $5HT_1$ receptors [2]. Second, the apparent dissociation constant determined for serotonin in blood vessels agreed with that found for the interaction of serotonin with $5HT_2$ but not $5HT_1$ receptors [2]. Third, there was an excellent correlation between the apparent dissociation constants of antagonists for serotonergic receptors in the vasculature to the IC_{50} for binding of the antagonists to $5HT_2$ receptors in brain cortical tissue ([3, 4] and Fig. 1).

* Send correspondence to: Dr. Marlene L. Cohen, Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285, U.S.A.

Departments of Cardiovascular Pharmacology and Central Nervous System Research
Lilly Research Laboratories
Eli Lilly & Co.
Indianapolis, IN 46285, U.S.A.

MARLENE L. COHEN*
NORMAN MASON
KATHRYN S. WILEY
RAY W. FULLER

REFERENCES

1. S. J. Peroutka and S. H. Snyder, *Molec. Pharmac.* **16**, 687 (1979).
2. M. L. Cohen, R. W. Fuller and K. S. Wiley, *J. Pharmac. exp. Ther.* **218**, 421 (1981).
3. J. E. Leysen, *J. Physiol., Paris* **77**, 351 (1981).
4. J. E. Leysen, C. J. E. Niemegeers, J. M. Van Nueten and P. M. Laduron, *Molec. Pharmac.* **21**, 301 (1982).
5. J. E. Leysen, F. Awouters, L. Kennis, P. M. Laduron, J. Vandenberk and P. A. J. Janssen, *Life Sci.* **28**, 1015 (1981).
6. J. M. Van Nueten, P. A. J. Janssen, J. Van Beek, R. Xhonneux, T. J. Verbeuren and P. M. VanHoutte, *J. Pharmac. exp. Ther.* **218**, 217 (1981).
7. C. W. Hooker, P. J. Calkins and J. H. Fleisch, *Blood Vessels* **14**, 1 (1977).
8. M. L. Cohen and K. S. Wiley, *J. Pharmac. exp. Ther.* **205**, 400 (1978).
9. R. F. Furchgott, *Ann. N.Y. Acad. Sci.* **139**, 553 (1967).
10. R. F. Furchgott, in *Catecholamines* (Eds. H. Blaschko and C. Muscholl), p. 283. Springer, Berlin (1972).
11. B. R. Zaborowsky, W. C. McMahon, W. A. Griffin, F. H. Norris and R. R. Ruffolo, *J. pharmac. Meth.* **4**, 165 (1980).
12. O. Arunlakshana and H. O. Schild, *Br. J. Pharmac Chemother.* **14**, 48 (1959).
13. D. L. Nelson, A. Herbet, S. Bourgoin, J. Glowinski and M. Hamon, *Molec. Pharmac.* **14**, 983 (1978).
14. J. P. Bennett, Jr. and S. H. Snyder, *Molec. Pharmac.* **12**, 373 (1976).
15. S. J. Peroutka and S. H. Snyder, *Brain Res.* **208**, 339 (1981).
16. D. N. Middlemiss, J. A. Carroll, R. W. Fisher and I. J. Mounsey, *Eur. J. Pharmac.* **66**, 253 (1980).
17. C. Ennis and B. Cox, *Neuropharmacology* **21**, 41 (1981).
18. S. J. Peroutka, R. M. Lebovitz and S. H. Snyder, *Science* **212**, 827 (1981).
19. S. Z. Langer and C. Moret, *Br. J. Pharmac.* **76**, 287P (1982).
20. M. A. Rogawski and G. K. Aghajanian, *J. Neurosci.* **1**, 1148 (1981).