Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 594-601

SPECIFIC BINDING OF [^{3}H] MEPYRAMINE TO HISTAMINE H_1-RECEPTORS IN THE SARCOLEMMA FROM PORCINE AORTA AND CORONARY ARTERY

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Received December 3, 1984

SUMMARY: We studied the subcellular distribution and the properties of [^{3}H] mepyramine binding in the porcine vascular smooth muscle. A close correlation was observed between the specific binding activity of [^{3}H] mepyramine and the extent of the enrichment of sarcolemmal marker enzyme in 4 subfractions obtained by sucrose density gradient centrifugation of the aortic microsome. In the binding isotherm in the sarcolemmal fractions from the aorta and coronary artery, there was no difference in the Kd value, but the Bmax of the coronary artery was significantly lower than that of the aorta. Thus, there is a single type of high affinity mepyramine binding site in the porcine vascular smooth muscle sarcolemma and the number of H_1-receptors of the coronary artery may be smaller than that of the aorta.

The effect of histamine on vascular smooth muscle has been debated, in relation to coronary artery spasm (1, 2). We recently induced angiographically demonstrable coronary artery spasm in miniature swine with experimentally-induced atherosclerotic lesions, by giving histamine, intravenously or intracoronarily (1). Pharmacological experiments showed that histamine initiates a contraction by an interaction with H_1-receptors and a relaxation by H_2-receptors (3, 4). Although histamine H_1-receptors in subcellular preparations of various peripheral target organs and brain (5-8) have been successfully characterized in binding assays by using its antagonist, [^{3}H] mepyramine, there were few reports in which [^{3}H] mepyramine binding was investigated in vascular smooth muscle (8, 9). In particular, in case of the coronary artery, no study of this type has been documented. We attempted to determine the characteristics of subcellular distribution of [^{3}H] mepyramine binding in porcine vascular smooth muscle, and to observe binding isotherms of this drug in sarcolemmal fractions isolated from the porcine aorta and coronary artery.

0006-291X/85 $1.50

MATERIALS AND METHODS

Preparations of the sarcolemmal fractions from aorta and coronary artery

Preparations of subcellular membrane fractions were essentially according to the method of Kwan et al. (10), with some modifications. For each preparation, 20 pig hearts or 5-6 pig aortas or 2 bovine aortas were obtained from a local slaughterhouse immediately after the animals had been killed and were transported to our laboratory in ice-cold buffer composed of 0.25 M sucrose, 10 mM MOPS (morpholinopropanesulfonic acid), and 0.05% bovine serum albumin, pH 7.4. The coronary arteries dissected from hearts and aortas were opened, longitudinally, the first intima layer was scraped off and then the media layer was stripped from the adventitia. The trimmed media layers were placed in 10 ml of buffer per gram of wet weight, finely minced, and homogenized with a Polytron PT 10 homogenizer at a setting of 7, twice for 8 seconds. The sample of the coronary artery was further homogenized by making 2 passes with a Teflon pestle and glass homogenizer at 400 rpm. The homogenate was centrifuged at 900g for 10 min, the supernatant was centrifuged at 9,000g for 20 min, and the supernatant was centrifuged at 108,000g for 45 min. The pellet was resuspended in 0.25 M sucrose solution containing 10 mM MOPS, pH 7.4 and re-centrifuged at 9,000 g for 10 min. The final supernatant (microsomal fraction) of the coronary artery was applied directly to the top of a discontinuous sucrose density gradient composed of 5.0 ml of 46.5% sucrose and 8.0 ml each of 38.5%, 32.5%, 26.5% sucrose solutions. In case of the aorta, the microsomal fraction was sedimented at 178,000g for 45 min and the pellet was resuspended in 5 ml of 46.5% sucrose and which formed the bottom of a discontinuous sucrose gradient described above. The gradient tube was centrifuged for 3 hours in case of the coronary artery and for 16 hours for the aorta, at 117,000g in a Hitachi SW 27 rotor. After centrifugation, the following 4 subfractions were obtained: F1; top layer and interfacial zone to 26.5% layer, F2; 26.5% layer and interfacial zone to 32.5% layer, F3; 32.5% layer and interfacial zone to 38.5% layer, F4; 38.5% layer and interfacial zone to 46.5% layer. Each subfraction was diluted and centrifuged at 178,000g for 45 min and final pellets were suspended in 0.25 M sucrose 10 mM MOPS solution pH 7.4 for enzyme assays and in 50 mM Na-K phosphate buffer pH 7.5 for binding assays. In case of binding assays for microsomes, the microsomal fractions from porcine and bovine aorta were sedimented at 178,000g for 45 min and the pellets were suspended in Na-K phosphate buffer, pH 7.5. All procedures were carried out at 4°C.

Enzyme assays

K^+-stimulated ouabain-sensitive p-nitrophenylphosphatase (K^+-pNPPase) and glucose-6-phosphatase activities were measured by the method of Bers (11). Cytochrome C oxidase was assayed according to the method of Cooperstein and Lazarow (12). NADPH cytochrome C reductase was assayed by the method of Kwan et al. (10). Protein was assayed according to Markwell et al., using bovine serum albumin as a standard (13).

Binding assays

Forty-120 μg protein of F2 fractions from the aorta or the coronary artery, or 150-200 μg protein of porcine aortic microsomes or 500-600 μg protein of bovine aortic microsomes were incubated at 25°C in a total volume of 1 ml containing 50 mM Na-K phosphate buffer, pH 7.5 with the indicated concentrations of [^{3}H] mepyramine for 30 min, with or without 2 μM promethazine, to determine the nonspecific or total binding, respectively. Binding was terminated by adding 5 ml of ice-cold buffer and filtered over Whatman GF/B glass fiber filters with 3x5 ml washes with ice-cold buffer. Liquid scintillation spectrometry was used for counting. Specific binding was defined as the total binding minus nonspecific binding. To determine the subcellular distribution of [^{3}H] mepyramine binding, about 100 μg protein of F1-F4 fractions from the aorta were incubated with 7 nM [^{3}H] mepyramine, with or without 2 μM promethazine. In the displacement studies, 80-120 μg protein of F2 or F3 fraction from the aorta and 1.5 nM [^{3}H] mepyramine were incubated with various concentrations of the indicated drugs, with or without 2 μM promethazine. The Ki values were calculated by the Cheng-Prusoff equation

(14). All binding assays were carried out in triplicate. The samples used were either fresh or frozen and were stored at -70°C up to 4 weeks. Freezing the samples and storing them up to 4 weeks did not significantly influence the specific binding.

Chemicals

[^{3}H] mepyramine (24.1 Ci/mmol) was obtained from New England Nuclear. Other reagents were of the highest grade commercially available.

Statistical methods

Group mean values were compared using Student's t-test, and results were considered significant when two tailed t-test demonstrated a $p<0.05$. Data are expressed mean ± S.E.

RESULTS

Marker distribution on subfractions (F1-F4) of the aorta and coronary artery is shown in Table 1. The activities of sarcolemmal marker enzyme, K^+-pNPPase, of both aorta and coronary artery had a peak at F2 fractions and were enriched about 14 fold and 20 fold from 900g supernatant of the homogenate in the aorta and coronary artery, respectively. Cytochrome C oxidase, a mitochondrial inner membrane marker, had a peak at F4 fractions in both the aorta and coronary artery. The activities of glucose-6-phosphatase and NADPH cytochrome C reductase, assumed to be markers for sarcoplasmic reticulum (11, 15), were not detected in any fraction of our preparations. Since there is no reliable marker for sarcoplasmic reticulum of vascular smooth muscle (16), the degree of the contamination of sarcoplasmic reticulum is difficult to determine. However, as the sarcoplasmic reticulum of vascular

Table 1. Distribution of enzyme activity in subcellular fractions

Fractions	K^+-pNPPase	Cyt. C oxidase
Aorta		
900g S.	0.0394 ± 0.006	0.0640 ± 0.002
F1	0.340 ± 0.038	0.0510 ± 0.051
F2	0.555 ± 0.037	0.125 ± 0.054
F3	0.530 ± 0.038	0.385 ± 0.009
F4	0.360 ± 0.031	0.494 ± 0.063
Coronary artery		
900g S.	0.0203 ± 0.0014	0.0933 ± 0.0051
F1	0.0801 ± 0.0103	0.134 ± 0.127
F2	0.413 ± 0.039	0.0483 ± 0.0483
F3	0.340 ± 0.031	0.240 ± 0.077
F4	0.0947 ± 0.0147	0.425 ± 0.105

Results are expressed in mean ± S.E. from three to four preparations. 900g S.; 900g supernatant of the homogenate. K^+-pNPPase; K^+-stimulated ouabain sensitive p-nitrophenylphosphatase expressed in μmol/mg protein/hour. Cyt. C oxidase; cytochrome C oxidase expressed in ΔO.D./mg protein/min.

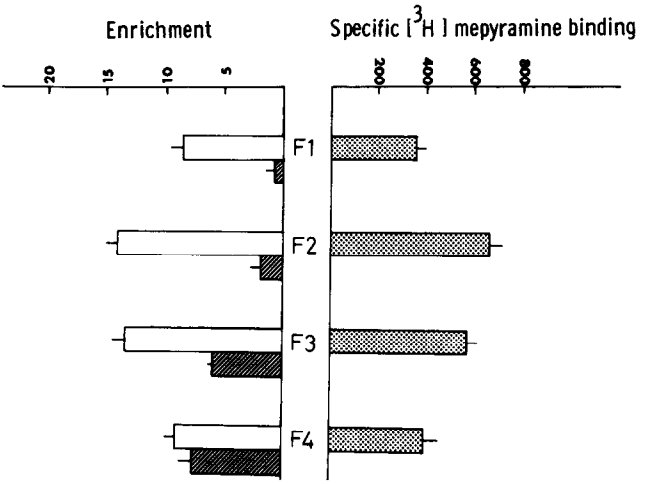

Figure 1 Subcellular distribution of biochemical markers (left) and specific [^{3}H] mepyramine binding (right) in smooth muscle of the porcine aorta. Biochemical markers (□; K^+-pNPPase, ▨; cytochrome C oxidase) are expressed in enrichment obtained by making the specific activities of these enzymes in 900g supernatant of the homogenate to be 1. Specific [^{3}H] mepyramine binding (▩) was expressed in fmol/mg protein at the [^{3}H] mepyramine concentration of 7 nM. The values are mean ± S.E. of three to four preparations of the marker enzymes and three preparations of specific [^{3}H] mepyramine binding. There is a good correlation between the sarcolemmal marker enzyme, K^+-pNPPase, and specific [^{3}H] mepyramine binding.

smooth muscle is poorly developed (17), the amount of contamination of sarcoplasmic reticulum, if any, is probably relatively small. Thus, we consider that the F2 fractions obtained from the aorta and coronary artery are almost equally and highly enriched subfractions composed predominantly of sarcolemma.

Figure 1 shows the distribution of the enrichment of 2 membrane marker enzymes and that of specific [^{3}H] mepyramine binding at the concentration of 7 nM in the subcellular fractions isolated from porcine aorta. A close correlation was observed between the specific binding of [^{3}H] mepyramine and the enrichment of K^+-pNPPase ($r=0.98$), but not so in the case of cytochrome C oxidase ($r=-0.09$).

Specific binding of [^{3}H] mepyramine in F2 fractions from aorta at 25°C was rapid and reversible, as shown in Figure 2. Scatchard analysis of the binding data gave the Kd of 1.29 ± 0.14 and 1.08 ± 0.08 nM, and the Bmax of 681 ± 57 and 310 ± 44 fmol/mg protein for aortic (n=4) and coronary (n=3) F2 fraction, respectively. A typical example is shown in Figure 3. Hill plot

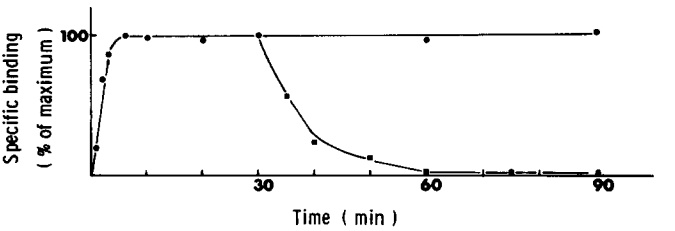

Figure 2 Association (●), and dissociation (■) kinetics of [^{3}H] mepyramine binding in the F2 fraction from porcine aorta. Dissociation kinetics was measured by adding 2 μM promethazine to sarcolemmal vesicles that had been incubated with 7 nM [^{3}H] mepyramine for 30 min at 25°C. Points are from 3 experiments with each point plotted individually. Each point was determined in triplicate. Association was rapid with half maximal specific binding ($t_{\frac{1}{2}}$) of 1 - 2 min, reaching equilibrium within 6 min. Binding remained stable for 90 min. Dissociation was rapid with a $t_{\frac{1}{2}}$ of 5 - 6 min.

analysis of these data resulted in a line with a slope of 0.94 ± 0.06 for the aorta (n=4) and 0.96 ± 0.02 for the coronary artery (n=3), indicating a single set of high affinity binding sites for this drug. In the displacement study, the specific binding of [^{3}H] mepyramine was inhibited in the following order of decreasing efficacy: promethazine, mepyramine, diphenhydramine, histamine, as shown in Figure 4. The Ki values were 0.54 ± 0.04 nM, 1.1 ± 0.1 nM, 11.7 ± 3.2 nM, 18 ± 5 μM, respectively (n=3-4). To investigate species differences

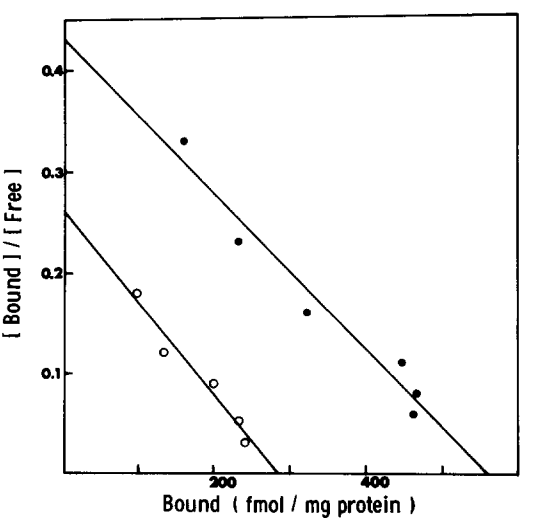

Figure 3 Scatchard plot of the data from binding isotherm in the F2 fractions from the aorta (●) and the coronary artery (o) as a function of increasing concentrations of added [^{3}H] mepyramine. The data represent findings in a typical experiment. Four independent experiments from 4 preparations were done for the aorta and 3 for the coronary artery.

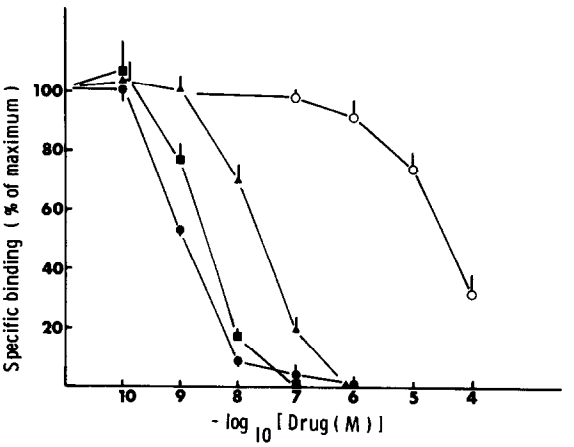

Figure 4 Effect of additions of promethazine (●), mepyramine (■), diphenhydramine (▲), and histamine (O) on specific [^{3}H] mepyramine binding at a concentration of 1.5 nM, on aortic sarcolemmal vesicles (F2 or F3 fraction), mean of 3 - 4 experiments. On the vertical axis data are expressed as a % of maximum specific [^{3}H] mepyramine binding. Vertical bars represent S.E. For this experiment, we used both the F3 and F2 fraction, because there was no difference in the inhibition patterns.

in the [^{3}H] mepyramine binding, we carried out the binding isotherm in microsomal fractions from porcine aorta and bovine aorta. As shown in Table 2, the Bmax of porcine aorta was much higher than that of bovine aorta.

DISCUSSION

The binding sites for [^{3}H] mepyramine are assumed to exist in the plasma membrane, however, detailed studies on the localization of specific [^{3}H] mepyramine binding have apparently not been documented. We found a good correlation between the high affinity specific [^{3}H] mepyramine binding and the enrichment of sarcolemmal marker enzyme K^+-pNPPase, suggesting that the sarcolemma is the locus of the high affinity [^{3}H] mepyramine binding. In the

Table 2. [^{3}H] mepyramine binding parameters for microsomal fractions from porcine aorta and bovine aorta

Species	Bmax	Kd
	fmol/mg protein	nM
Porcine	387 ± 57 (n = 4)*	1.36 ± 0.13 (n = 4)
Bovine	72 (n = 2)	1.22 (n = 2)

The values are mean ± S.E. or mean (n preparations).
*$P < 0.05$, compared to the value in bovine.

displacement study, the inhibition of specific [^{3}H] mepyramine binding was in generally good agreement with observations in binding studies (6, 8, 9, 18) and also in pharmacological experiments using intact tissues (19 - 21). The reversibility seen in kinetic studies, the localization of specific [^{3}H] mepyramine binding, saturability in binding isotherm, and good correlation between [^{3}H] mepyramine binding and pharmacological characteristics in competitive studies support the conclusion that the histamine H_1-receptor can be labelled with [^{3}H] mepyramine, with a high affinity, in porcine vascular smooth muscle. In the equilibrium binding study, the Kd values of [^{3}H] mepyramine binding in F2 fractions from aorta and coronary artery were similar and also much the same as the values reported by other investigators. (3 nM for beef aorta (9), 4.0 nM for guinea pig aorta (8), 1.5 nM for rabbit aorta (8), 1.35 nM for guinea pig trachea (6), and 1.6 nM for guinea pig intestine (5)). The Bmax value of [^{3}H] mepyramine binding in F2 fractions of the coronary artery was significantly lower than that of the aorta ($p < 0.01$). Since the F2 fractions of both aorta and coronary artery appeared to be enriched to an almost equal extent, the lower Bmax value might indicate a lower density of the H_1-receptors in the coronary artery. In addition, the present study on microsomal fractions demonstrates that the Bmax of porcine aorta is about 5 times higher than that of bovine aorta. The Bmax of bovine aorta (72 fmol/mg protein) is of a similar order of the values reported by other investigators who used different species of animals for homogenate or crude pellets for bovine (9), guinea pig, and rabbit aorta (8) (31, 59, and 27 fmol/mg protein, respectively). The possibility that the density of H_1-receptors in the porcine coronary artery may be higher than that of other species has to be given due attention as the vessels in this species closely resemble those in man (22).

Histamine induces coronary artery spasm in miniature swine with experimentally-induced atherosclerotic lesions (1). Whether there is a relatively high density of H_1-receptors in the coronary artery and whether there are changes in the affinity or density of H_1-receptors in the presence

of atherosclerosis in miniature swine, are the subjects of ongoing studies in our laboratories.

ACKNOWLEDGEMENTS

We are grateful to M. Ohara for critical reading of the manuscript and to A. Nishi and N. Hayashi for secretarial services. This study was supported in part by grants for Scientific Research Nos 58870058 and 59440044 from the Ministry of Education, Science and Culture Japan.

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