[^{3}H]NOREPINEPHRINE BINDING: UNRELATED TO CATECHOL-O-METHYL TRANSFERASE

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Received April 29,1974

SUMMARY

[^{3}H]norepinephrine binds *in vitro* to microsomal membranes derived from a wide variety of tissues. Controversy exists as to the physiological significance of this binding phenomenon and recently the suggestion has been made that this represents binding to the enzyme catechol-o-methyl transferase (COMT). The enzyme and the [^{3}H]norepinephrine binding sites however, are shown to have very different characteristics. Tissues having the highest levels of microsomal catechol-o-methyl transferase(liver, kidney) bind the least [^{3}H]norepinephrine. The enzyme is concentrated in soluble fractions of tissue whereas the binding sites are found almost exclusively in membrane fractions. Purified preparations of the binding sites have no catechol-o-methyl transferase activity. pH optima, K_M for epinephrine, and substrate specificity are all strikingly different for the enzyme and the [^{3}H]norepinephrine binding sites. It is concluded that *in vitro* [^{3}H]norepinephrine binding is unrelated to microsomal catechol-o-methyl transferase.

Reports from a number of laboratories have described the characteristics of *in vitro* binding of [^{3}H]catecholamines to membranes derived from heart (1,2), liver (3,4), turkey erythrocytes (5,6) spleen capsule (7) and fat (8) as well

as to intact myocardial cells(9,10). The physiological significance of this binding has been the subject of some controversy (11,12,13). In particular, this has focused on whether the binding sites are related to physiological adrenergic receptors, neuronal or other uptake processes, degradative enzymes previously undefined uptake sites, or some combination of these processes. The nonidentity of the sites with previously defined neuronal and non-neuronal uptake processes has been documented elsewhere (14).

Recently, Cuatrecasas and colleagues have contended that *in vitro* [^{3}H] catecholamine binding is a reflection of interaction with membrane bound catechol-o-methyl transferase (COMT)[1] (12,13). This conclusion was based on the specificity of both [^{3}H]norepinephrine binding and COMT for the catechol ring; the inhibitory effects of COMT inhibitors on [^{3}H]norepinephrine binding; and a putative enhancing effect of S-adenosyl methionine (a cofactor of COMT) on [^{3}H]norepinephrine binding (12).

It is the purpose of this communication to: 1) present data which refute this hypothesis and 2) point out several observations concerning [^{3}H]norpinephrine binding reported by Cuatrecasas et al which cannot be confirmed in this laboratory.

If *in vitro* [^{3}H]norepinephrine binding to membrane fractions and intact cells is a reflection of interaction with COMT, then several consequences should follow. First, enzyme and binding activity should have comparable tissue and subcellular distributions. Accordingly, we examined tissue and subcellular distribution of both activities. Table 1 demonstrates data obtained when COMT and [^{3}H]norepinephrine binding activity were compared in the microsomal fractions from 5 canine tissues. No correlation between the two activities was observed. In fact, the tissues which had the highest levels of microsomal COMT (liver, kidneys) had the lowest [^{3}H]norepinephrine binding activity and vice versa. This pattern was consistent in each of the 5 animals studied. As noted

[1] The abbreviation used is COMT = catechol-o-methyl transferase

TABLE 1

TISSUE from which microsomes prepared	[^{3}H]NOREPINEPHRINE BOUND cpm/mg protein	[^{3}H]METANEPHRINE FORMED cpm/mg protein	[^{14}C]METANEPHRINE FORMED cpm/mg protein
Skeletal Muscle	24,400±3100	22,680±2600	17,470±2100
Cardiac Muscle	22,900±2700	22,620±2500	22,870±2200
Spleen	14,750±2200	30,650±2700	38,910±3600
Kidney	14,600±1800	72,820±3600	40,920±4100
Liver	10,750±2100	104,800±9400	45,450±5300

[^{3}H]norepinephrine binding and catechol-o-methyl transferase activity in microsomes from various tissues. Microsomal membranes were prepared from 5 mongrel dogs after sacrifice by intravenous pentobarbital. Tissues were immediately removed and placed in ice cold 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4. Each tissue was handled separately and all operations were performed at 0-4 C°. Tissues were minced with scissors then homogenized with 10 up and down strokes of a Teflon tipped, motor driven homogenizer. Tissue homogenates were spun for 10 minutes at 10,000 xg in a Sorvall RC2B centrifuge and the supernatant then centrifuged at 105,000 xg for 1 hour in a Beckman L-265B ultracentrifuge. The microsomal pellets so obtained were resuspended in the sucrose buffer and recentrifuged once or twice. Membranes were stored frozen at -20°C and assayed for binding or COMT within 2 weeks. Both activities were unaffected by these periods of freezing. Proteins were determined by the method of Lowry.

[^{3}H]norepinephrine binding was assayed by procedures similar to those reported previously (1,2). d,l-[^{3}H] norepinephrine (10 Ci/mmole), New England Nuclear Co., Boston, Massachusetts), 10^{-8}M was incubated with membranes,

Table 1 cont.

generally 50-100 μg membrane protein in 1 ml 0.1 M phosphate buffer, pH 7.4 for 30 minutes at 37° C. [^{3}H]norepinephrine bound to sites in the membranes was then determined by rapid Millipore filtration (0.45 μ pore size HAWP 02500) followed by a 10 ml buffer wash. Controls were included in all experiments in which incubations were performed in the presence of 10^{-4}M unlabelled norepinephrine. The small number of counts retained on the Millipore filters under such circumstances (∿0.3% of the counts added) was subtracted from all experimental determinations. Values shown are the mean ± SE of triplicate determinations on membranes from 5 different animals.

Catechol-o-methyl transferase was assayed by 2 separate methods. Method 1 was exactly as described by Axelrod (19) and followed the conversion of [^{3}H]epinephrine to [^{3}H]metanephrine in the presence of S-adenosyl methionine. The second method, also described by Axelrod (20) followed the formation of [^{14}C]metanephrine from epinephrine in the presence of [^{14}C]S-adenosyl methionine (10 mCi/mmole, New England Nuclear Company). Enzyme assays were performed at pH 7.4. Values shown for [^{3}H]metanephrine formed are means ± SE of duplicate determinations on membranes from 5 animals, whereas those for [^{14}C]metanephrine are values for membranes from 3 animals.

in the table, the enzyme determinations were performed by 2 different assay methods.

When soluble fractions and crude membrane fractions were compared for COMT and binding activity, almost no binding activity was found in the soluble fraction whereas COMT was high in this fraction (Table 2). Conversely, binding

TABLE 2

TISSUE FRACTION	[^{3}H]NOREPINEPHRINE BOUND cpm/mg protein		[^{14}C]METANEPHRINE FORMED cpm/mg protein	
	pH 7.0	pH 7.8	pH 7.0	pH 7.8
Microsomal Membranes	5700±610	9200±910	42,300±5100	14,700±1300
105,000 xg supernatant	480±120	750±90	34,300±3700	32,600±2600

[^{3}H]norepinephrine binding and COMT in membranes and supernatant fractions from canine livers. All procedures were performed as described in legend to Table 1. Values are means ± SE triplicate determinations from membranes prepared from two animals.

activity was concentrated in the membranes whereas COMT was lower in these fractions. Also, it is possible that some of the COMT activity in the microsomes was in fact soluble enzyme which was adsorbed to the membrane. It was regularly observed that microsomal COMT was higher at pH 7.0 than at pH 7.8. [^{3}H] norepinephrine binding on the other hand was significantly inhibited at the lower pH, as previously reported (2). These findings are in agreement with the subcellular distribution of [^{3}H]epinephrine binding in liver previously found by Marinetti et al (3). When solubilized preparations of [^{3}H]norepinephrine binding protein (cardiac) purified by affinity chromatography by previously described methods

(15) were tested for COMT activity, none was found.

A second result to be expected if [^{3}H]norepinephrine binding were in fact related to COMT, is that dose response curves for enzyme and binding activities would reveal comparable K_M's. As shown in Figure 1, this is not the case. 1/2 maximal displacement of [^{3}H]norepinephrine from binding sites was observed with

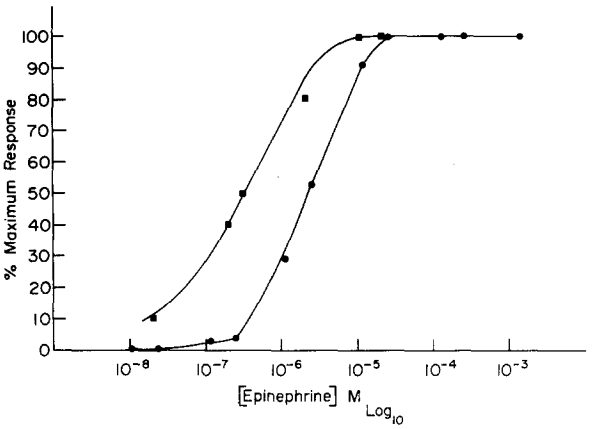

Fig. 1 Concentration dependence of a) epinephrine inhibition of [^{3}H] norepinephrine binding and b) COMT activity in cardiac microsomal membranes. Methods were as previously described (Method II was used for COMT assay). Values are means of 3 experiments. ●—● COMT activity. ■—■ inhibition of [^{3}H]norepinephrine binding.

epinephrine at 3 x 10^{-7}M, a concentration at which almost no COMT activity was observed. 1/2 maximal COMT activity did not occur until a 10 fold higher concentration of epinephrine was reached.

A third expectation if [^{3}H]norepinephrine binding to microsomal membranes was related to COMT, would be parallel substrate specificities for both processes. As noted above, both COMT and [^{3}H]norepinephrine binding sites show a major specificity for the catechol function. However, the specificities are not identical. Soterenol may be used as an example of the divergent specificities of the two processes. This beta agonist resembles isoproterenol save that the OH, on the 4 position of the ring, is replaced by a CH_3SO_2NH function. We found that this compound does not serve as a substrate for

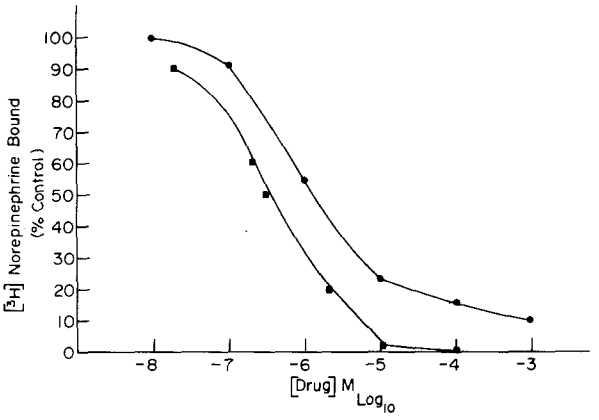

Fig. 2 Inhibition of [^{3}H]norepinephrine binding to cardiac microsomal membranes by epinephrine and soterenol. Values shown are means of triplicate determinations in 2 experiments. ■—■ epinephrine; ●—● soterenol.

microsomal COMT from heart at concentrations tested up to 10^{-3}M. By contrast, as noted in Figure 2, it effectively competed with [^{3}H]norepinephrine for binding to sites in the cardiac membranes. 1/2 maximal inhibition of binding was observed at about 10^{-6}M which is somewhat less potent than either epinephrine or norepinephrine (1). It should be noted that these findings are in contrast to those of Cuatrecasas et al (11) who found that soterenol did not compete effectively for binding to liver or heart microsomes.

Another feature of the data reported by Cuatrecasas et al (12) which could not be confimred in this laboratory is the putative effect of S-adenosyl methionine (SAM) on [^{3}H]norepinephrine binding. These authors reported that SAM at 0.1-1 mM strikingly increased both the rate and extent of [^{3}H]norepinephrine binding to liver and heart membranes. We have studied the effects of 3 separate lots of SAM at comparable concentrations on the rate and extent of binding of [^{3}H]norepinephrine to fresh and frozen cardiac microsomes at 23° and 37°. 10^{-4}M SAM had no effect whereas 10^{-3}M SAM caused either no effect or slight (up to 30%) inhibition. The reason for these contradictory findings is not clear, though species difference (rat vs. dog) may play a role. However,

any conclusions that SAM is related to *in vitro* [^{3}H]norepinephrine binding seems unwarranted, at this time.

Cuatrecasas et al (12) also reported that COMT inhibitors such as pyrogallol and tropolone inhibited [^{3}H]norepinephrine binding to liver membranes. This is presumably a reflection of the major specificity of both binding and COMT for the catechol ring. Belleau et al (16) have previously shown that inhibitors of COMT such as tropolone are biochemically "isosteric" with the catechol ring in a variety of systems (16) including, apparently, at some beta adrenergic receptors (17). In this context it should be noted that Bilzekian and Aurbach had previously reported that pharmacologically inert catechol compounds such as dihydroximandelic acid inhibited isoproterenol activated adenylate cyclase in turkey erythrocyte membranes, though they were considerably less active in intact cells (5). Cuatrecasas et al (12,13) reported an opposite set of observations, namely that catechol compounds did not inhibit isoproterenol activated cyclase in turkey erythrocyte membranes, except at very high concentrations. Curiously, the discrepant findings of Bilzekian and Aurbach have not been noted by Cuatrecasas and his colleagues (12,13).

Although the physiological significance of *in vitro* [^{3}H]catecholamine binding to membranes from a variety of tissues remains a matter of active investigation and debate, the data presented here clearly indicate that this binding is not related to COMT. The enzyme and the binding sites have different tissue distributions, are concentrated in different subcellular fractions, have differing K_M's and nonidentical specificities. In addition, putative effects of the COMT cofactor SAM could not be confirmed.

ACKNOWLEDGEMENT

The author wishes to thank Mr. Michael Coverstone for his excellent technical assistance. This work was supported by N. I. H. grant #RO1-HL 16037 01 and by a grant-in-aid from the American Heart Association with funds contributed in part by the North Carolina Heart Association. Dr. Lefkowitz is an Established Investigator of the American Heart Association..

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