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RE-EVALUATION OF THE RELATIONSHIP BETWEEN CATECHOL-*O*-METHYL TRANSFERASE AND THE BINDING OF NOREPINEPHRINE TO BROWN ADIPOCYTE MEMBRANES

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Abstract—The binding of labeled norepinephrine to brown adipose tissue intact microsomes or solubilized microsomal proteins was studied *in vitro*.

The effects of incubation in oxygenated Krebs–Ringer bicarbonate buffer, pH 7.4, on the physicochemical state of norepinephrine was investigated. After 10 minutes of incubation, the recovery of norepinephrine (0.5 μM) by alumina adsorption technique was found to be only about 40 per cent and no oxidation was detected by polarography. The recovery could be increased to over 90 per cent by adding metal chelators to the Krebs–Ringer bicarbonate buffer or by incubation in a phosphate buffer, which suggests that contaminating metals can cause considerable complexation of the hormone. The assays with unmodified norepinephrine were therefore performed under the following conditions: 10 min incubation in 50 mM phosphate buffer, pH 7.4, 25°.

In both intact microsomes and solubilized microsmal proteins, the binding of norephinephrine was found to be sensitive to substances affecting catechol-*O*-methyl transferase activity such as tropolone, normetanephrine, dithiothreitol, Ca^{2+} and Ca^{2+} chelators; agents that inhibited catechol-*O*-methly transferase activity were shown to stimulate norepinephrine binding and *vice versa*.

After separation of solubilized microsomal proteins by Ultrogel AcA 34 filtration, both norepinephrine binding and catechol-*O*-methly transferase activity were found in the same protein fraction. Separation by polyacrylamide gel electrophoresis revealed congruent migration of norepinephrine binding, catechol-*O*-methyl transferase activity and *S*-adenosyl methionine binding.

It is now well established that the β-adrenergic receptors could be responsible for only a very small percentage of the binding, observed *in vitro*, of labeled catecholamines to membrane proteins [1–8]. Two hypotheses to explain the bulk of this binding have been proposed. According to the first [1, 2], it is the result of an interaction between oxidized catechol and membrane proteins and therefore of no biological significance. A second hypothesis suggests that it could be due to an interaction of labeled catecholamines and catechol-*O*-methyl transferase (COMT, EC 2.1.1.6)* in an altered state [3]. The purpose of this study was to evaluate these hypotheses in an attempt to discern a possible biological significance of NE binding.

Since catechols in solution are known to be readily oxidized under aerobic conditions and have been found to form very stable complexes with metals [9–12], a careful investigation was made to determine the extent to which oxidation or complexation might modify the physicochemical state of NE in solution. In order to test the second hypothesis, NE binding sites and COMT were partly purified and the NE binding and COMT activity were determined under conditions allowing for valid comparison.

MATERIALS AND METHODS

Materials

DL-[7-^{14}C]Norepinephrine (20–40mCi/mmole), DL-[7-^{3}H]norepinephrine (5–15 Ci/mmole) and *S*-adenosyl-L-[methyl-^{3}H]methionine(40–60mCi/mmole) were purchased from New England Nuclear, Frankfurt, Germany. Catechol analogues of NE, NM and SAM were obtained from Sigma Chemical Co., St. Louis, Mo., L-DL-NE from Fluka, Buchs, Switzerland, DTT from Calbiochem., Los Angeles, U.S.A. and tropolone from K and K Laboratories Inc., New York. All other chemicals (reagent grade) were obtained from Merck, Darmstadt, Germany.

All solutions were prepared with bidistilled water. Radioactivity was measured in a Beckman scintillation counter.

Methods

Preparation of intact microsomes and solubilized microsomal proteins. Sprague–Dawley male rats, weighing about 200 g, were decapitated and the interscapular BAT was excised. The microsomal fraction, containing mostly plasma membrane, was prepared as

* Abbreviations used are: BAT: brown adipose tissue, COMT: catechol-*O*-methyl transferase, DDT: dithiothreitol, EDTA: ethylenediamine tetraacetic acid, EGTA: ethyleneglycolbis (β-aminoethyl ether) *N,N'*-tetraacetic acid, KRBB: Krebs–Ringer bicarbonate buffer, NE: norepinephrine, NM: normetanephrine, PO_4: phosphate, SAM: *S*-adenosyl methionine, Tiron: pyrocatechol-3,5-disulfonic acid.

described previously [13]. The microsomes were solubilized at 4° by suspension and homogenization in PO_4 buffer 50 mM, pH 7.4, containing deoxycholate (0.25%). The solution was centrifuged at 105.000 *g* for 1 hr. The supernatant was frozen in liquid nitrogen and stored at −20°. Proteins were measured by the method of Lowry *et al.* [14].

Binding assay. The binding of labeled NE was measured by filtration [2] after 10 min of incubation at 25° in PO_4 buffer 50 mM, pH 7.4. Nonspecific binding was determined after the addition of an overload of unlabeled NE. All assays were performed in duplicate. The measurements of NE binding to solubilized proteins were confirmed by microdialysis, indicating that the solubilized proteins were quantitatively adsorbed on the filters.

NE assay. Unmodified NE recovered after incubation under standard conditions was measured by the alumina adsorption technique [15, 16].

Polarographic measurements. The stability of NE in solution was measured by normal pulse polarography on dripping mercury, using the electrochemistry system PAR No 170.

COMT assay. COMT activity was measured according to the method of Axelrod [17]. About 0.1 mg of protein was incubated for 10 min at 25° in PO_4 buffer 50 mM, pH 7.4, $MgCl_2$ 1 mM, DTT 1 mM, NE 50 μM, [methyl-^{3}H]SAM 0.2 μCi (50 μM). The reaction was stopped by adding 0.25 ml of borate buffer 0.4 M, pH 10. Blank values were determined by omitting NE. All assays were performed in duplicate. Labeled NM was extracted in toluene-isoamyl alcohol (3:2), and its radioactivity determined. The purity of the NM was verified by alumina thin layer chromatography (solvent: acetate buffer 0.2 M, pH 8.4).

Gel filtration. About 2 mg of solubilized proteins were placed in a column (100 × 0.8 cm), filled with Ultrogel AcA 34 equilibrated with PO_4 buffer 50 mM, NaCl 0.1 M, pH 7.4 at 4°. The proteins were eluted with this buffer at a flow rate of 14 ml/hr and 2 ml fractions were collected. In one type of experiment, the solubilized proteins were labeled by incubation with L-[7-^{3}H]NE (0.5 μM) under the conditions used in the binding assay and the excess free ligand was removed by dialysis. They were then placed in the column and the radioactivity of each eluted fraction was counted. In another type of experiment, unlabeled proteins were eluted under identical conditions except for the addition of 1 mM DTT and 1 mM $MgCl_2$ to the buffer and the COMT activity of each eluted fraction was measured.

Gel electrophoresis. The Ultrogel-eluted proteins were submitted to Triton X 100 polyacrylamide gel electrophoresis at 4° [18, 19]. Some of the gels were coloured with amido black. In one type of experiment, proteins were labeled as described above with either DL-[7-^{14}C]NE (5 μM) or [methyl-^{3}H]SAM (1 μM), dialysed, then submitted together to electrophoresis. The gels were cut in 2 mm slices in a Gilson gel mincer and the ^{3}H and ^{14}C content of each slice was determined. In another type of experiment, unlabeled proteins were submitted to electrophoresis with 1 mM DTT and 1 mM $MgCl_2$ added to the gels and buffer. The gels were then cut and the COMT activity of each slice was measured.

RESULTS

Analysis of NE after incubation in saline solutions. The modifications of the physicochemical state of NE after incubation in saline solutions are illustrated in Table 1. It can be seen, first of all, that after 10 min of incubation in KRBB containing no biological material, only about 40 per cent of the 0.5 μM of NE was recovered unmodified. Polarographic measurements made to determine whether this modification was due to oxidation show that the NE was not oxidized after 20 min of bubbling with oxycarbon (95% O_2–5% CO_2). Furthermore, addition of trace amounts of Cu^{2+} to the polarographic buffer did not catalyze oxidation. It can also be seen in Table 1 that the addition of 50 μM Cu^{2+} completely modified the 100 μM of NE initially present in the solution, indicating the formation of the complex CuL_2. Complexation with contaminating metal could, therefore, account for the considerable modification of NE (0.5 μM) in the KRBB. Overloads of glycine or various agents known to be highly effec-

Table 1. Physicochemical state of NE

	% Unmodified NE recovered
Alumina separation (NE 5 μM), incubation 10 min	
KRBB	39
KRBB + glycine 10^{-3} M	66
KRBB + tiron 10^{-5} M	62
KRBB + DOPA 2.5×10^{-5} M	83
KRBB + Vit. C 2.5×10^{-5} M	79
PO_4 buffer 10^{-2} M	94
Polarography (NE 10^{-4} M), incubation 20 min	
$NaHCO_3$ buffer + oxycarbon	98
+ Cu^{2+} 5.10^{-6} M	90
+ oxycarbon + Cu^{2+} 5.10^{-6} M	90
+ Cu^{2+} 5.10^{-5} M	0

NE was incubated in the indicated media at pH 7.4. Analytical techniques are described under Methods. Results are expressed in per cent of NE added to medium (mean of 3 experiments).

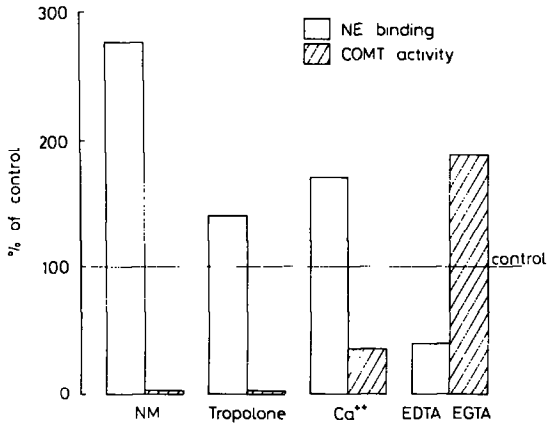

Fig. 1. Effects of various agents on NE binding and COMT activity in solubilized microsomal proteins. NE binding and COMT activity were measured as described. Tropolone was 2.10^{-4}M; NM, Ca^{2+} or divalent cation chelators were 10^{-3}M each. Results are the mean of 4 determinations, expressed in per cent of control value (no addition) for NE binding or COMT activity, respectively.

tive metal chelators were found, in fact, to result in increased recovery of unmodified NE. It was also found that in a PO_4 buffer (50 mM, pH 7.4) whose concentration of free metals is insignificant, the recovery of unmodified NE was almost total.

The PO_4 buffer and short incubation periods (10 min) were therefore chosen for studying NE binding to intact microsomes and solubilized microsomal proteins.

Binding and its relationship to COMT. Binding of unmodified NE to microsomes or solubilized proteins was found to be slow (equilibrium not yet reached after 1 hr incubation) and saturation was not reached with concentrations of NE ranging from 10^{-8} to 5×10^{-5}M. Various catechol analogues, as well as D- and L-NE, were found to cause a decrease in L-NE binding, which was found to be unaffected by propranolol. These observations confirm that the β-receptors are not involved in the bulk of NE binding.

Control experiments were performed in order to verify that NE actually did bind to adipocyte membranes and not to endothelial cell or nerve ending membranes. NE binding was found not to be affected by reserpine (an inhibitor of NE neuronal storage) or by desipramine (an inhibitor of NE neuronal uptake) [20]. Furthermore, results for microsomes prepared from isolated adipocytes or from denervated BAT were essentially the same as those obtained for the controls.

Results of tests of binding reversibility made with intact microsomes showed that after 45 min incubation in an NE-free PO_4 buffer, 42 per cent of the bound radioactivity had been released. Similar tests made with solubilized microsomal proteins, on the other hand, showed that after 18 hr of dialysis, there was no change in bound radioactivity. A loss of radioactivity (about 40 per cent) was observed only when the PO_4 buffer contained 1 mM each of DTT, SAM and Mg^{2+}.

Several substances known to affect COMT activity were tested for their effects on NE binding to solubilized microsomal proteins. Figure 1 shows the results obtained for: tropolone, a specific COMT inhibitor,

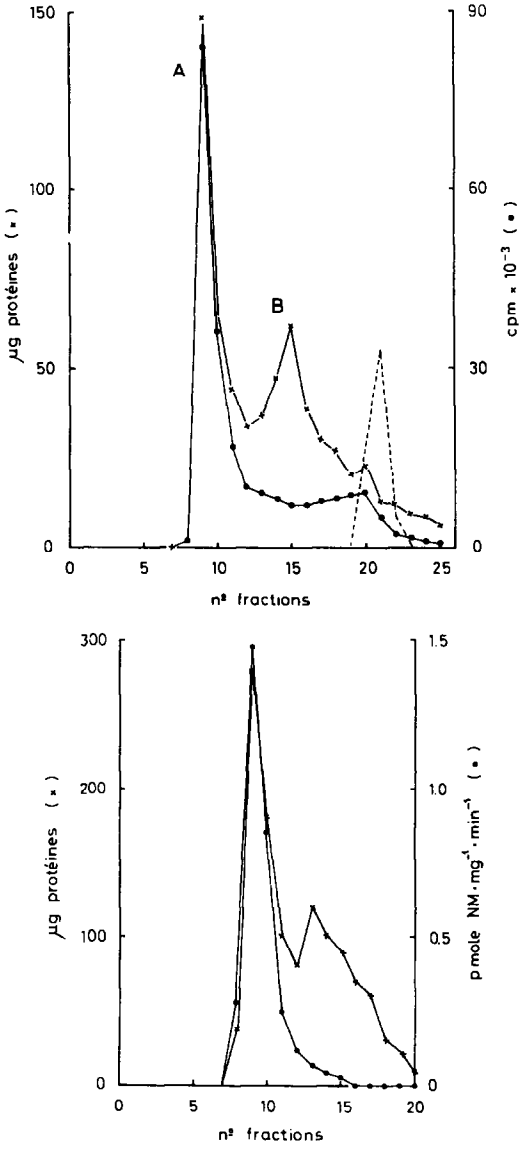

Fig. 2. Ultrogel AcA 34 filtration of solubilized microsomal proteins. The experiments were performed as described. Upper panel: elution curve of NE-labeled proteins; dotted line: control elution of NE alone (1 μCi ^{3}H NE + 5 mg NE). Lower panel: elution curve of COMT activity.

NM, the product of NE methylation, Ca^{2+} and EDTA. Similar results were also obtained for intact microsomes. These same agents were also tested in assays of enzyme activity (Fig. 1), except that EDTA was replaced by EGTA, since COMT requires free Mg^{2+} as cofactor. It can be seen that the agents that inhibit COMT activity stimulate NE binding and vice versa. It was also found that NE binding decreased and COMT activity increased as a function of increasing concentrations of DTT. In fact, there was not detectable COMT activity in the absence of DTT with binding unaffected, and conversely, there was not detectable binding when COMT activity was maximal (10^{-3} M DTT).

Characterization of NE binding and COMT activity by means of protein separation. Solubilized microsomal proteins were fractionated on Ultrogel AcA 34. Peak A illustrated in Fig. 2 corresponds to the recovered radio-

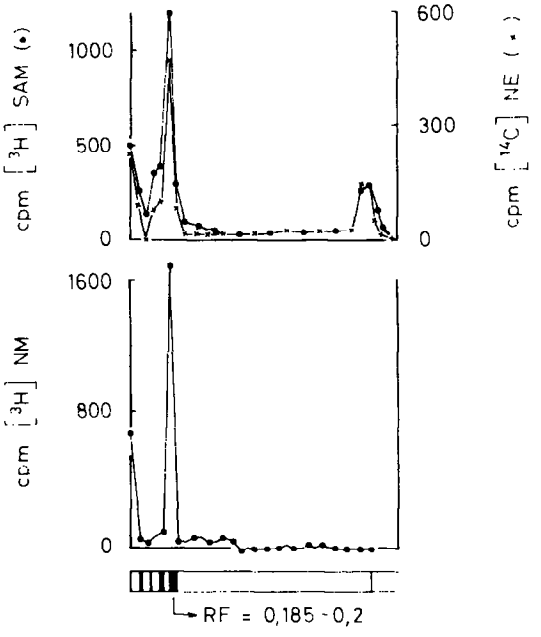

Fig. 3. Triton X-100 polyacrylamide gel electrophoresis of solubilized proteins. The experiments were performed as described, with Ultrogel-eluted proteins (peak A, Fig. 2). Upper panel: ^{3}H SAM- or ^{14}C NE- labeled proteins (dual labelling). Lower panel: COMT activity. Amido black-coloured protein bands are shown at the bottom.

activity of bound NE, as well as COMT activity. Analysis by SDS polyacrylamide gel electrophoresis revealed that peak A comprised only four of the 15 protein bands detected in the crude solubilized microsomal protein preparation.

In order to determine which of these four protein bands corresponds to COMT and which to the NE binding, Ultrogel-eluted proteins were labeled with either ^{14}C NE or with ^{3}H SAM (since it had been noted that labeled SAM could bind to solubilized proteins) and submitted to Triton X-100 polyacrylamide gel electrophoresis. Figure 3 demonstrates not only that the radioactivity peaks of bound SAM and bound NE are congruent, but also that they correspond to the same protein band as COMT activity.

DISCUSSION

The present attempt to reevaluate the various hypotheses concerning *in vitro* binding of NE to membranes has, first of all, confirmed the finding that the β-receptors, which have been successfully identified by means of non-catechol artificial ligands [21–27], are definitely not involved quantitatively in the binding of unmodified natural hormone to microsomes or solubilized microsomal proteins in BAT of the rat.

The hypothesis explaining catechol binding as an interaction between oxidized catechol and membrane proteins [1, 2] was not verified, since no oxidation of NE was detected, probably because of the much shorter incubation period used in the present study. It was found, however, that a decrease in NE concentration did occur after 10 min incubation in KRBB but this was almost certainly the result of complexation of NE by contaminating metal traces, since it could be prevented by the addition of metal chelators such as glycine or tiron to the medium. Catecholamine–metal complexation in biological saline solutions must, therefore, be taken into account in evaluating dose-response curves. *In vitro* physiological experiments performed in this laboratory [28] have shown, in fact, that the respiratory response of BAT to NE was increased about three-fold by adding tiron, which, by itself, does not induce a respiratory response, to the medium.

The second hypothesis, i.e. that NE binding might be related to COMT [3], is supported by the results of the present study. The effects on NE binding of substances affecting the activity of COMT suggests the following description of its functioning: in an active state of the enzyme, there would be not detectable binding of NE, while in an inactive state there would be NE binding which would be irreversible. Results obtained by means of protein separation technique lent further support to the hypothesis; it was found that only one and the same protein band corresponded not only to the binding of NE and SAM (both COMT substrates) but also to COMT activity. Previous investigators of this hypothesis [2, 29–31] concluded that NE binding and COMT activity were unrelated phenomena, probably because a direct relationship had been expected.

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