ENHANCED SPECIFICITY OF EPINEPHRINE BINDING BY RAT LIVER PLASMA MEMBRANES IN THE PRESENCE OF EDTA

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Received April 15,1974

SUMMARY

Rat liver plasma membranes bind tritiated epinephrine by a time and temperature dependent, saturable process. The binding of epinephrine was readily displaced by all compounds possessing a catechol moiety, unless 1 mM EDTA was added to the assay mixture. Under these conditions, EDTA addition diminished the number of binding sites and pyrocatechol, 3-4 dihydroxymandelic acid and tropolone could no longer compete with the labelled ligand. It is postulated that the effect of EDTA occurs *via* chelation of a divalent cation. Addition *in vitro* of EDTA to the assay mixture seems therefore to obviate part of the non-specificity which hampers investigation of the "catecholamine receptor" in liver.

While considerable progress was achieved recently in the identification of membrane receptors for polypeptide hormones and cholinergic agents, studies of the catecholamine receptor (1 - 8) have been thus far hampered by an apparent lack of specificity of binding even though other criteria (saturability, time and temperature dependence) were fulfilled. In all the systems studied, the binding of labelled catecholamines by membrane preparations was inhibited by compounds possessing a 3-4 dihydroxy phenolic moiety but devoid of any biological activity (1, 2, 5, 7). The "catechol-binding protein" studied was considered to be either a structure related to the enzyme catechol-O-methyl transferase (COMT)* (7) or only a part of the putative catecholamine "receptor" (5 - 6).

Data reported here indicate that the addition of the chelating agent EDTA markedly enhances the specificity of the epinephrine binding to isolated rat liver plasma membranes. Binding could no longer be displaced, under these conditions, by compounds possessing only the catechol moiety.

*Abbreviation used: COMT: *S*-Adenosyl-L-methionine: Catechol-*O*-methyltransferase (E.C. 2.1.1.6).

EXPERIMENTAL PROCEDURE

Materials: The following were obtained as gifts: propranolol from I.C.I.; β desoxy-isoproterenol, d- and l-epinephrine bitartrate from Sterling Winthrop Research Institute; N-isopropyl metoxamine HCl from Burroughs Wellcome Co. l-isoproterenol, l-phenylephrine HCl, dihydroxy mandelic acid and pyrocatechol were purchased from Sigma. Tropolone was from Regis. EDTA (ethylene diamine tetra-acetic acid) was from Merck. [7-^{3}H] dl-epinephrine (9-10 Ci/mmole) was obtained from New England Nuclear Corp. It was systematically checked for purity by thin layer chromatography on cellulose plates with the following solvent system: *n*-butanol-acetic acid-water (50:15:25, v/v/v). The developed spots were visualized by the *p*-nitroaniline procedure. Labelled epinephrine was used only when purity was close to 100 %.

Methods.

Plasma membrane preparation. Plasma membranes were prepared from liver of adrenalectomized, female, Wistar rats (about 100 g body weight) according to the procedure devised by Neville (9) up to step 11. The purified membrane preparations were suspended in 1 mM $NaHCO_3$ and stored up to six weeks in liquid nitrogen without any loss of activity. Protein was estimated by the Lowry's procedure (10) using bovine serum albumin as standard.

Assay for binding of [7-^{3}H] epinephrine to liver membranes. Liver plasma membranes (1 mg protein/ml) were incubated at 37° for various time periods in an assay medium containing 1 mM EDTA and 50 mM Tris-HCl, pH 7.4 in a final volume of 120 µl. The reaction was initiated by addition of [7-^{3}H] epinephrine at the final concentrations indicated in the legends to figures. Three to five determinations were made for each assay. At the end of the incubation, a 100 µl aliquot was filtered through a Millipore filter (HAWP 02500) and washed twice with 5 ml of ice-cold Tris buffer (50 mM, pH 7.4). The filtration and the washing steps were done in less than 15 sec. The filters were then placed in scintillation vials, eluted for 10 min with 1 ml of 1 N acetic acid and finally added with 13 ml of Unisolve (Koch Light Laboratory) for determination of the radioactivity. Blanks were performed by previous addition of 1 mM

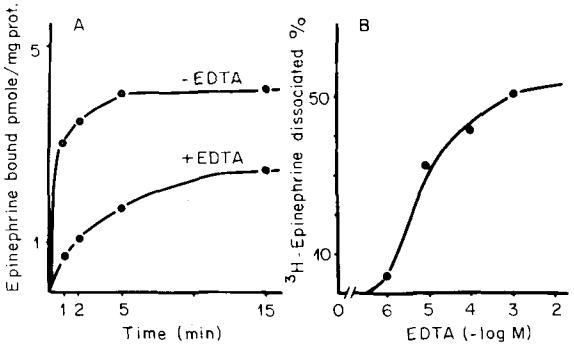

Fig. 1a. Time course of [^{3}H] epinephrine binding to rat liver plasma membranes with and without 1 mM EDTA. Incubation at 37° with or without 1 mM EDTA was initiated by addition of 0,5 µM epinephrine (specific activity was about 1700 cpm/pmole). Aliquots of 100 µl were removed at the indicated time points and [^{3}H] epinephrine binding determined as described under "methods". Each point is the mean of triplicate determinations.

Fig. 1b. Effect of EDTA concentration on [^{3}H] epinephrine binding. Membranes were incubated at 37° in 50 mM Tris buffer (pH 7.4) for 3 minutes with 5 µM epinephrine (specific activity about 230 cpm/pmole). Varying concentrations of EDTA in a negligible volume were then added and 3 min later [^{3}H] epinephrine binding was determined as described under "methods".

unlabelled epinephrine to the plasma membranes; the amount of radioactivity in these samples was subtracted to calculate specific binding. In order to reduce binding of tritiated epinephrine to the filters, they were soaked for 20 min prior to use in the dark, in an ice-cold Tris buffer (50 mM, pH 7.4) containing 1 mM unlabelled epinephrine.

RESULTS

As shown in Fig. 1a, the binding of labelled epinephrine by isolated rat liver plasma membranes was a time-dependent process and the maximal binding was attained in a few minutes (3-5 minutes in the absence of EDTA and 10 minutes in its presence). This figure reveals also that addition of 1 mM EDTA decreased the total binding by about 50 %. This inhibitory action was dose-dependent: as depicted in Fig. 1b, maximal effect occurred with 1 mM EDTA. Addition of Mg, in the absence of EDTA, did not change the binding pattern. It should be noted that, under our conditions, the binding of epinephrine to rat liver plasma

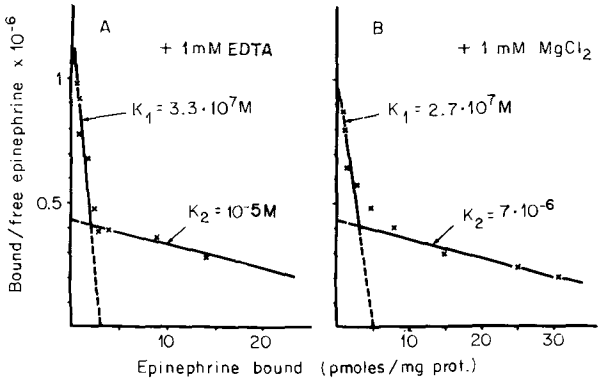

Fig. 2a and 2b. Scatchard plots for epinephrine binding to rat liver plasma membranes. Various amounts of unlabelled epinephrine from 50 nM to 10 µM were added to incubation medium containing membranes, tritiated epinephrine (100 000 cpm) and 1 mM EDTA or Mg. After 15 min of incubation at 37°, binding was determined as described under "methods".

membranes was a rather fast phenomenon, as compared to the other systems described (1, 5).

The binding process was saturable. Fig. 2a and 2b represent the Scatchard plots (11) of the binding of [^{3}H] epinephrine after incubation for 15 minutes in the presence of 1 mM EDTA or Mg respectively. These plots suggest that there are probably two orders of sites having apparent dissociation constants of about 3.10^{-7}M and 10^{-5}M, corresponding respectively to 3 and 50 picomoles of epinephrine bound per mg of membrane protein. In the presence of 1 mM Mg instead of EDTA, the K_D were 3.10^{-7}M and 7.10^{-6}M, corresponding respectively to 6 and 60 picomoles of epinephrine bound per mg protein. The only apparent effect of EDTA was to decrease the number of sites with high affinity. The figures reported here for the K_D and sites numbers are in good agreement with the ones found in other systems (3, 8). Moreover, the lower K_D (3.10^{-7}M) is close to the concentration of epinephrine (8.10^{-7}M) giving half maximal activation of the adenylate cyclase in our system (unpublished data).

With regard to the specificity of binding, and as many others (1, 2, 5, 7), we observed that the only requirement for various compounds to displace the epinephrine binding, in the

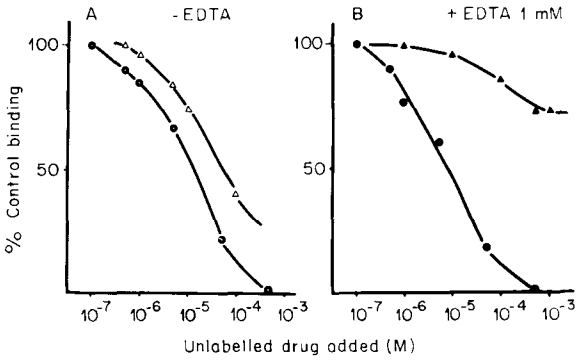

Fig. 3a and 3b. Effect of varying concentrations of cold epinephrine and pyrocatechol upon the binding of [^{3}H]epinephrine in the absence (a) and the presence (b) of 1 mM EDTA. The incubation was initiated by addition of 0.1 µM [^{3}H]epinephrine immediately before pyrocatechol (△,▲) or cold epinephrine (○,●) at the indicated concentrations. After 3 min at 37°, epinephrine binding was determined as described under "methods."

absence of EDTA, was the existence of a catechol group. For example, as shown in Fig. 3a, pyrocatechol, which has no physiological activity, was found to be as effective as epinephrine in competing for the binding of tritiated epinephrine. However, in the presence of 1 mM EDTA, pyrocatechol did not displace the binding by more than 25 % even though its concentration was increased 10^4 fold, whereas epinephrine was still as effective.

This finding prompted us to study the specificity of binding of epinephrine in the presence of 1 mM EDTA. As depicted in Fig. 4, binding of labelled epinephrine was not displaced, or only to a limited extent, by compounds such as dihydroxy mandelic acid, pyrocatechol or tropolone that would be otherwise quite efficient. Under these conditions, maximal dissociation of the residual binding occured only with epinephrine and isoproterenol. The d and l forms of epinephrine competed similary with [^{3}H] epinephrine while β-desoxy isoproterenol was slightly less effective than isoproterenol.

DISCUSSION

Binding of epinephrine with rat liver plasma membranes is a time-dependent and saturable process. In the experiments reported here, membranes from adrenalectomized rats were routinely used

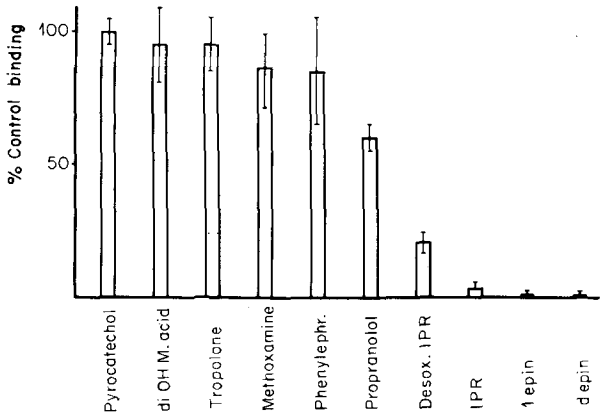

Fig. 4. Effects of various compounds on [^{3}H] epinephrine binding. Incubation was initiated by addition of 5 µM [^{3}H] epinephrine with unlabelled compounds (final concentration 0.5 mM) and carried out for 2min at 37°. (IPR = Isoproterenol, diOH M. acid = diOH mandelic acid). The bars indicate the SEM (five determinations per assay).

since they are much more sensitive to epinephrine for the adenylate cyclase activation (12-14). The main point which we would like to emphasize concerns the enhanced specificity of this binding brought about by the presence of EDTA in the assay mixture, as compared to the experiments where no EDTA was used, in the present work (Fig. 3a) as well as with other systems (1, 2, 5, 7). In the presence of the chelating agent, the catechol moiety was no longer sufficient to compete with epinephrine binding (Fig. 3b and 4).

This effect of EDTA is likely to be related to chelation of a divalent cation. It is thus possible that part of the binding of epinephrine, with no EDTA added, is due to the presence of COMT in the membranes, for the presence of a divalent cation is well known to be necessary for the enzymic activity (15). Assay for COMT in our membranes revealed that about 0,1 % of the total liver COMT activity is present in the plasma membrane fraction (unpublished data). That the residual binding, in the presence of EDTA, is no longer displaced by pyrocatechol or tropolone suggests that it is not linked to the COMT but is rather possibly more directly related to the hypothetical catecholamine "receptor". The lack of stereospecifity for the binding of epinephrine reported here (Fig. 4) as well as in all the previous studies (1, 2, 5, 7) obviously precludes any premature identification of the binding

site with the actual catecholamine receptor. However, as reported here for the first time, the lack of specificity of the catechol moiety can be obviated with the use of a chelating agent. This should be considered encouraging for further investigation toward isolation of the epinephrine receptor.

ACKNOWLEDGMENTS

We are grateful to D. Stengel for technical assistance and to Dr P. Berthelot for helpful and valuable discussion of this study. This work was supported by the "Institut National de la Santé et de la Recherche Médicale", the "Délégation Générale à la Recherche Scientifique et Technique" and by the "Fondation pour la Recherche Médicale".

REFERENCES

1. Lefkowitz R. J. and Haber E. (1971) Proc. Natl. Acad. Sci. U.S.A., 68, 1773-1777.
2. Schramm M., Feinstein H., Naim E., Lang M. and Lasser M., (1972) Proc. Natl. Acad. Sci. U.S.A., 69, 523-527.
3. Fiszer-de-Plazas, S. and de Robertis, E., (1972) Biochim. Biophys. Acta, 266, 246-254.
4. Lefkowitz, R. J., Sharp, G. W. G. and Haber, E., (1973) J. Biol. Chem., 248, 342-349.
5. Bilezikian, J. P. and Aurbach, G. D., (1973) J. Biol. Chem., 248, 5575-5583.
6. Bilezikian, J. P. and Aurbach, G. D., (1973) J. Biol. Chem., 248, 5584-5589.
7. Cuatrecasas, P., Tell, G. P. E., Sica, V., Parikh, I. and Chang, K. J., (1974) Nature, 247, 92-97.
8. Lefkowitz, R. J., O'Hara, D. and Warshaw, J., (1973) Nature New Biol., 244, 79-80.
9. Neville, D. M., (1968) Biochim. Biophys. Acta, 154, 540-552.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., (1951) J. Biol. Chem., 193, 265-275.
11. Scatchard, G., (1949) Ann. N. Y. Acad. Sci., 51, 660-669.
12. Bitensky, M. W., Russel, V. and Blanco, M., (1970) Endocrinology, 86, 154-159.
13. Leray, F., Chambaut, A. M. and Hanoune, J., (1972) Biochem. Biophys. Res. Commun., 48, 1385-1391.
14. Leray, F., Chambaut, A. M., Perrenoud, M. L. and Hanoune, J., (1973) Eur. J. Biochem., 38, 185-192.
15. Axelrod, J. and Tomchick, R., (1958) J. Biol. Chem., 233, 702-705.