EPINEPHRINE BINDING BY RAT LIVER PLASMA MEMBRANES: EFFECT OF GUANYL NUCLEOTIDES.

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SUMMARY. In purified plasma membranes from rat liver, GTP stimulated the epinephrine sensitive adenylate cyclase and promoted the dissociation of the bound hormone from its binding sites. However, unlike the data reported with respect to glucagon, the two effects of GTP upon the epinephrine sensitive cyclase and the binding of epinephrine did not appear to be causally related. The GTP action on epinephrine binding was mimicked by EDTA as well as by other nucleotides devoid of any effect upon the cyclase activity. The GTP action was reversed by adding Mg^{2+} to the incubation medium and was clearly due to a simple chelation phenomenon. Conversely GDP displaced only poorly the bound hormone although it was a good stimulator of the cyclase. GTP appears, therefore, to exert dual and unrelated effects upon the epinephrine sensitive adenylate cyclase: it displaces epinephrine from its binding sites *via* a chelation phenomenon whereas it influences directly the catalytic component of the cyclase system.

Activation of adenylate cyclase by guanyl nucleotides, synergically with many stimulating hormones, has been described in various tissues (1-8). Moreover, Rodbell *et al* (1, 9) reported that rat liver plasma membrane system could respond to GTP by a simultaneous increase in the cyclase activity in response to glucagon and in the dissociation of this hormone from its binding sites. These authors concluded that these two effects were either closely related (10) or interdependent (11). The same membranes possess an epinephrine-sensitive adenylate cyclase, which is markedly enhanced by guanyl nucleotides (2, 3). They possess also binding sites for epinephrine (12). The aim of the present study was, therefore, to investigate whether the addition of GTP would dissociate the bound epinephrine, in order to assess the possible link between these two intriguing effects of GTP. We provide here evidence that GTP dissociates about the half of bound epinephrine by a chelation phenomenon unrelated to its activating effect upon the cyclase activity.

EXPERIMENTAL PROCEDURE

MATERIALS. EDTA and $MgCl_2$ were obtained from Merck. Nucleotides (disodium salts), various guanyl derivatives and *l*-epinephrine bitartrate were from Sigma. Cyclic AMP and creatine phosphate were from Calbiochem; creatine kinase was from Boehringer. Bovine serum albumin (fraction V) was from Armour Pharmaceutical Company. [7-^{3}H] *dl*-epinephrine (9-10 Ci/mole) was obtained from New England Nuclear Co. 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 %. [α-^{32}P] ATP (500-900 mCi/mmole) and cyclic [8-^{3}H] AMP (13 Ci/mmole) were obtained from the CEA (Saclay, France).

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 (13) 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 (14) using bovine serum albumin as standard.

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Krishna *et al* (15), as modified by Pohl *et al* (16). Labelled cyclic AMP was isolated according to Ramachandran (17) and the yield calculated by previous addition of [8-^{3}H] cAMP. Incubation and sample counting were performed as previously described (3), except that no EDTA nor EGTA were added in the incubation medium.

Assay for binding of [7-^{3}H]epinephrine to liver membranes. Liver plasma membranes (1 mg protein/ml) were incubated at 37°C for various time periods in an assay medium containing 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

Abbreviations: EDTA: Ethylenediamine Tetraacetic Acid; EGTA: Ethyleneglycol-*bis*(β-aminoethyl Ether)-N, N'-tetraacetic Acid.

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

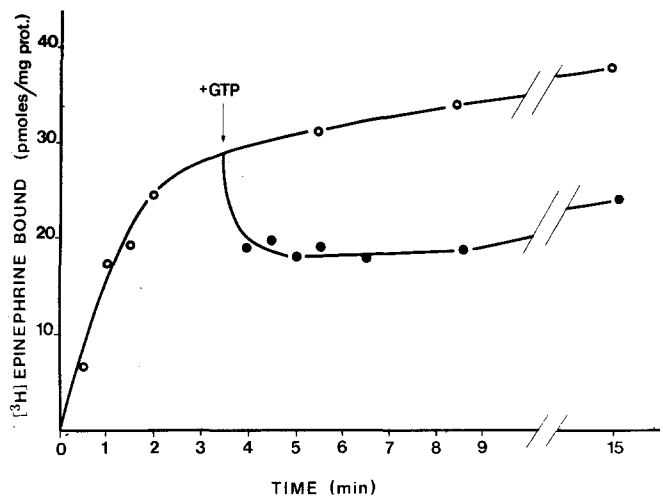

Fig. 1. Effect of addition of GTP on the time course of the [^{3}H] epinephrine binding. Assay system as described in Experimental Procedure. 0.1 mM GTP was added in a negligible volume 3 min and a half after the addition of 5 µM labelled epinephrine (about 220 cpm/pmole) to the membranes. Binding was determined as described under "methods" at the indicated time points. (○) control; (●) after addition of 0.1 mM GTP.

RESULTS. We previously reported that epinephrine binding to purified rat liver plasma membrane was a saturable, temperature and time dependent phenomenon (12). This binding is characterized by two orders of dissociation constant (0.3 µM and 10 µM) corresponding respectively to 3 and 50 picomoles of epinephrine

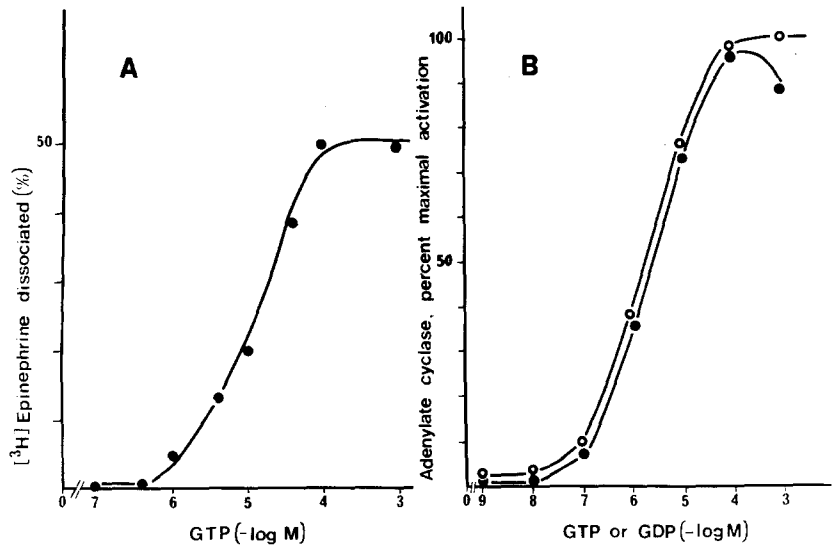

Fig. 2 A. Effect of varying concentrations of GTP on the dissociation of the bound [^{3}H] epinephrine from liver plasma membranes. Membranes were incubated for 3 min in 50 mM Tris buffer (pH 7.4) with 5 µM labelled epinephrine (about 220 cpm/pmole). Varying concentrations of GTP were then added in a negligible volume and 3 min later [^{3}H] epinephrine binding was determined as described under "methods".

Fig. 2 B. Effect of GTP and GDP upon the cyclase activity in the presence of epinephrine. Membranes were incubated for 10 min with the usual assay medium, 10 µM epinephrine and varying concentrations of GTP (●) and GDP (○).

bound per mg of membrane protein. The same membrane preparation possesses an adenylate cyclase sensitive to epinephrine (half maximal activation obtained with 0.8 µM epinephrine), synergically with GTP (3). As shown in Fig. 1, GTP acted upon the epinephrine binding in a way apparently similar to that described by Rodbell *et al* for glucagon (9). Binding of epinephrine to the liver plasma membranes attained a plateau after three minutes of incubation. The addition of 0.1 mM (final) of GTP led to an immediate dissociation of about 40 % of the bound hormone.

This effect of GTP was dose-dependent: a 5 % displacement of the bound radioactivity occurred with 1 µM GTP (Fig. 2 A) and the maximal effect was obtained at 0.1 mM. We never observed a complete release of the labelled hormone even with higher concentrations of GTP. The dose-dependent, synergic, effect of GTP on the epinephrine sensitive cyclase is depicted in Fig. 2 B.

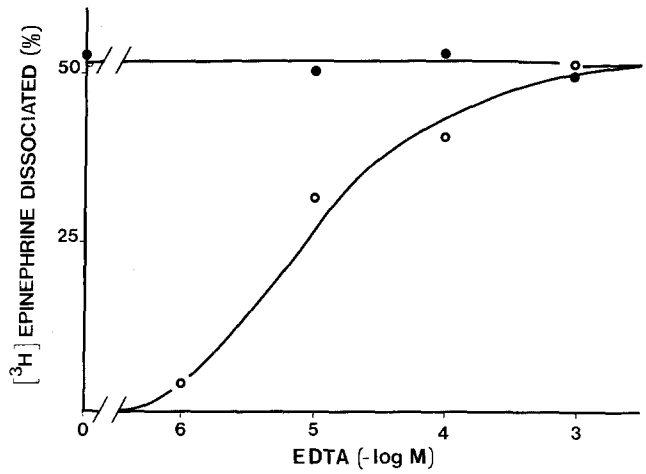

Fig. 3. Effect of varying concentrations of EDTA on [^{3}H] epinephrine binding in the presence or not of 0.1 mM GTP. EDTA at the stated concentrations was added in a negligible volume with (●) or without (○) 0.1 mM GTP after the beginning of the incubation, as described in the legend to Fig. 2 A.

As previously reported (2), this compound is a strong stimulator of the cyclase activity. It would therefore appear that a correlation might exist between the two effects of GTP upon the epinephrine stimulated adenylate cyclase and the release of the bound hormone from its binding sites.

However, the minimal response of the cyclase to GTP was obtained with a concentration of GTP lower by two orders of magnitude than the concentration necessary to promote dissociation of the bound hormone (Fig. 2 A and 2 B). Moreover, in a similar range of concentrations, EDTA was found nearly as efficient as GTP in promoting the release of bound epinephrine. Maximal dissociation was attained with 1 mM EDTA and was similar to that observed with 0.1 mM GTP (compare Fig. 3 with Fig. 2 A). Simultaneous addition of GTP and EDTA, both at concentration giving maximal dissociation, had no cumulative effects (Fig. 3). Conversely, addition of 1 mM EDTA to the adenylate cyclase assay mixture brought about a further enhancement of the enzyme response to 10 µM GTP and 50 µM epinephrine.

That GTP might dissociate the labelled epinephrine by virtue of a chelation phenomenon was further established

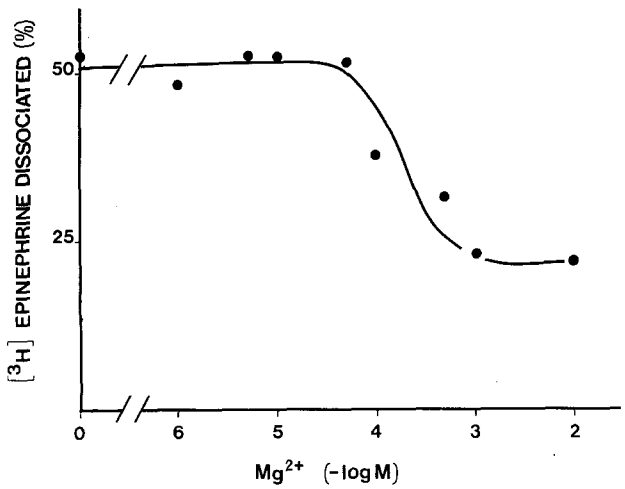

Fig. 4. Effect of Mg^{2+} on the [^{3}H] epinephrine binding in the presence of 0.1 mM GTP. Mg^{2+} (●) was added as described in the legend to Fig. 2 A in the presence of 0.1 mM GTP.

by the experiment depicted in Fig. 4: adding increasing amounts of Mg^{2+} to GTP reversed its action on dissociation. This effect of Mg^{2+} was no longer observed when concentration of EDTA equimolar to Mg^{2+} was also present. As shown in Table 1, similar effects on the hormone binding were obtained with other triphospho-nucleotides such as dGTP, ATP, ITP and even UTP, which was absolutely ineffective in enhancing the cyclase activity (3). GDP, which was as efficient as GTP upon the cyclase, (Fig. 2 B) was quite inefficient upon the epinephrine dissociation. Guanosine, cyclic GMP and guanosine diphosphoglucose did not alter the hormone binding. This is not surprising since mono- and diphospho-nucleotides bind divalent cation much less than triphospho-nucleotides.

DISCUSSION. It is likely that GTP acts upon the dissociation of epinephrine *via* chelation of divalent cations (not obligatorily Mg^{2+}) which would be involved in the binding of the hormone (12). An enhanced degradation of GTP by nucleotidase activity in the presence of Mg^{2+} or Ca^{2+} does exist (18) and could explain the opposite effects of GTP and Mg^{2+}. But, it is clearly not involved in the phenomena described above (Fig. 4) since the effect of GTP

upon the adenylate cyclase activation occurs in the presence of a large excess of Mg^{2+} (2 mM) in the assay medium (2, 3)). Our data emphasize that the effect of GTP on glucagon and epinephrine binding by rat liver plasma membranes is not a single mechanism. With respect to glucagon, Rodbell and coworkers (1, 9, 10) actually demonstrated that GTP could dissociate the bound hormone by a phenomenon clearly different from a chelation since EDTA was always present in the assay mixture, acting in a permissive way. Conversely, the inability of GTP to displace bound isoproterenol from turkey erythrocyte membranes, as recently reported by Bilezikian and Aurbach (19), is most probably due to the presence of 5 mM Mg^{2+} in their assay medium. Chelating agents such as EDTA (12) or triphospho-nucleotides (as described here) affect a part of the epinephrine binding to rat liver plasma membranes which probably involves divalent cations. Guanyl nucleotides influence probably the cyclase activity through a direct action on the catalytic site of the enzyme (3). Their effect upon the dissociation of hormones appears therefore a secondary (glucagon) or an unrelated (epinephrine) phenomenon.

Compound	[^{3}H]epinephrine dissociated (%)
ITP	50 ± 2
CTP	48 ± 8
UTP	41 ± 4
ATP	37 ± 4
GTP	43 ± 2
Guanosine-5'tetra phosphate	34 ± 3
dGTP	35 ± 6
GDP	8 ± 5
GMP	9 ± 3
Guanosine	6 ± 5
Cyclic GMP	5 ± 3
GDP-glucose	0

Table 1. Effect of various nucleotides and guanyl derivatives upon the [^{3}H]epinephrine binding. Membranes were incubated as indicated in the legend to Fig.2 A; various compounds were added in a negligible volume at the final concentrations of 0.1 mM. Binding was measured as described under "methods". Results are depicted as mean ± SEM (five determinations per assay).

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