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PROTOPORPHYRIN IX ACTIVATES THE Mg DEPENDENT GUANYLATE CYCLASE FROM RAT LIVER PLASMA MEMBRANES

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SUMMARY: In the presence of Mg-GTP, the rat liver guanylate cyclase, in either intact membranes or trypsin solubilized form, was stimulated by protoporphyrin IX 6 to 10-fold. However, when Mn-GTP was the substrate, protoporphyrin IX activated the membrane-bound guanylate cyclase only 50 %, in contrast to the marked activation reported for the cytosolic enzyme. Meso- and deuteroporphyrin IX, hematoporphyrin and coproporphyrin III also activated membrane guanylate cyclase while uroporphyrin III, and hemin had no effect. Basal, Mg^{2+}-dependent activity exhibited two classes of catalytic sites with apparent Km values of 2 mM and 0.12 mM. Activation by protoporphyrin resulted in the dissapearance of the low affinity sites. The activated enzyme exhibited Michaelis-Menten kinetics and no alteration in its requirement for excess Mg^{2+}. These data indicate that, in the presence of Mg^{2+}, a heme-like structure can interact with the membrane-bound guanylate cyclase and regulate its activity.

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.) generally exists in both soluble and particulate fractions of cell homogenates (for reviews see 1 and 2). It has not yet been determined which form of guanylate cyclase regulates the intracellular level of cyclic GMP (2, 3).

Compounds, which are capable of forming NO, markedly elevate cyclic GMP levels in several tissues and activate the cytosolic guanylate cyclase (4-7). These effects might be relevant to the smooth muscle relaxant and platelet antiaggregatory properties of these compounds (8, 9).The cytosolic guanylate cyclase has been purified to homogeneity (10-12) and contains a heme moiety (13), which is apparently not activator per se but is required for the activation by NO and related agents (14, 15). The cytosolic enzyme can be directly activated by the heme precursor, protoporphyrin IX (16, 17). This suggests that NO acts by modifying the iron-heme interaction, inducing an active protoporphyrin like-planar structure (16).

The abbreviations used are: NO, nitric oxide; cyclic GMP, cyclic guanosine 3',5'-monophosphate.

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The particulate enzyme has been only partially purified (18, 19). There was no or little activation by nitrosocompounds (7, 18, 20). In order to examine whether this lack of activation may be due to altered interaction of heme with the membrane enzyme, we studied the direct effect of protoporphyrin IX on the guanylate cyclase, either membrane-bound or after solubilization by trypsin (21). The activity was measured in the presence of the preferential *in vitro* substrate, Mn-GTP and also in the presence of Mg-GTP, which is thought to be the natural substrate (1). The trypsin-solubilized guanylate cyclase possesses a higher specific activity than that of the native, membrane-bound enzyme (22) and contains nearly no GTPase activity (22). Hence, it can be conveniently used to measure the low, Mg-dependent activity, as well as for a kinetic characterization of the enzyme.

In this communication, we present evidence that protoporphyrin IX increases the Mg^{2+}-dependent activity of membrane enzyme to the level of the Mn^{2+}-dependent activity. This regulation might be relevant to physiological control of cyclic GMP level.

MATERIALS AND METHODS

Materials. Commercial reagents used for the guanylate cyclase assay were those previously described (22). Protoporphyrin IX, hematoporphyrin, mesoporphyrin IX dimethylester, coproporphyrin III tetramethylester, uroporphyrin III octamethylester, hemin, bilirubin, trypsin and soybean trypsin inhibitor were obtained from Sigma. Deuteroporphyrin IX was purchased from Koch-Light Laboratories. The commercially available methyl ester derivatives of protoporphyrins were found to be inactive on guanylate cyclase. They were therefore hydrolyzed prior to use according to Falk (23).

Enzyme preparation. Plasma membranes were prepared from livers of female, albino Wistar rats (about 100 g body weight) according to the procedure of Neville (24) up to step 11 and stored in liquid nitrogen.

Proteolytically solubilized guanylate cyclase was prepared from membranes (5 mg/ml) previously washed with 50 mM Tris-HCl pH 7.6 after thawing and then treated by trypsin (2 µg/mg membrane protein) as previously described (22). A soluble form of guanylate cyclase was obtained with a Mr of 140.000 (21).

Guanylate cyclase assay. Guanylate cyclase activity was measured as described by Levilliers et al (25) with minor modifications (20). The reaction mixture contained 50 mM Tris-HCl, pH 7.6, 1 mM [alpha-^{32}P]GTP (1 to 2 µCi), 1 mM [8-^{3}H] cyclic GMP (10^4 cpm), 5 mM dithiothreitol, 3 mM either $MgCl_2$ or $MnCl_2$, bovine serum albumin (0.5 mg/ml), 15 mM creatine phosphate and 1 mg/ml creatine kinase in a final volume of 50 µl. The reaction was started by adding the enzyme solution (5 to 10 µg/assay) and performed for 20 min at 37°C unless otherwise indicated. Cyclic GMP was isolated as previously described (20). All values represent means of triplicate determinations which agreed within ± 5 %. Protein concentration was determined by the method of Lowry et al (26) using bovine serum albumin as standard.

RESULTS

The effect of increasing concentrations of protoporphyrin IX was tested on either the membrane-bound or the trypsin-solubilized guanylate cyclase. The activity was measured in the presence of either Mg^{2+} or Mn^{2+} together with GTP (Fig. 1). In the presence of Mg^{2+}, a 500 % and 800 % activation with $S_{0.5}$ for protoporphyrin IX of 0.2 and 2 μM was observed for the trypsin solubilized and the membrane-bound guanylate cyclase, respectively. With Mn-GTP as the substrate, the activation was only 50 % for both enzymes. No detectable guanylate cyclase activity was observed in the presence of protoporphyrin and in the absence of metal, thus ruling out the possibility that the activation was due to metal contamination of protoporphyrin IX.

Activation of the Mg^{2+}-dependent activity by precursors and derivatives of protoporphyrin IX is shown in Table I. Reduction of the two vinyl groups of protoporphyrin IX (mesoporphyrin IX) or their replacements by hydroxyethyl groups (hematoporphyrin) did not modify the extent of activation. However, deuteroporphyrin IX, in which the two vinyl groups are replaced by H, was less active. Of the two oxidized intermediates in the biosynthesis of protoporphyrin (uro-and coproporphyrin III) only coproporphyrin

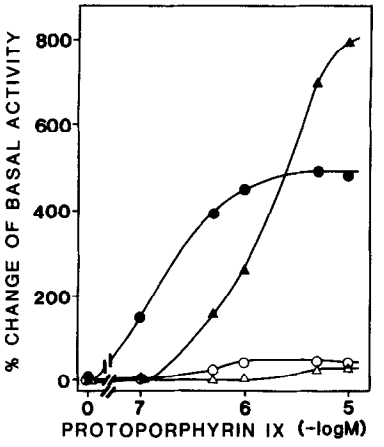


Figure 1: Effect of protoporphyrin IX on trypsin solubilized and membrane-bound guanylate cyclase activities. Trypsin solubilized (○ , ●) and plasma membrane (△ , ▲) fractions were tested for guanylate cyclase activity as indicated under "Materials and Methods" in the presence of 3 mM excess Mn^{2+} (○ , △) or Mg^{2+} (● , ▲) over 1 mM Me^{2+}-GTP. Basal activities were 2 and 5 pmol/mg/min when measured with Mg^{2+} and 20 and 30 pmol/mg/min with Mn^{2+}, for the membrane-bound and solubilized activities, respectively.

TABLE 1: EFFECT OF VARIOUS PRECURSORS AND DERIVATIVES OF PROTOPORPHYRIN IX UPON THE Mg^{2+}-DEPENDENT GUANYLATE CYCLASE OF THE NATIVE OR TRYPSIN SOLUBILIZED MEMBRANES.

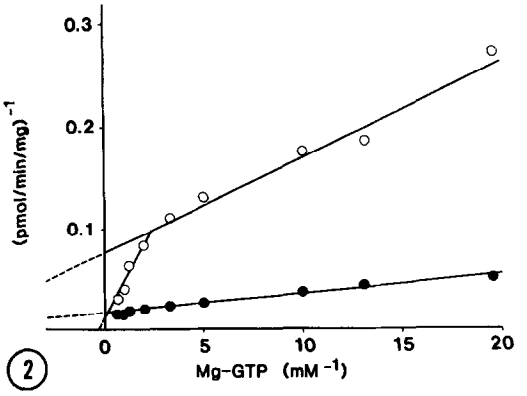
	GUANYLATE CYCLASE ACTIVITY pmol/mg prot/min	
ADDITION	MEMBRANE-BOUND	TRYPSIN SOLUBILIZED
None	2 ± 1	5 ± 2
Protoporphyrin IX	18 ± 4	54 ± 3
Mesoporphyrin IX	19 ± 1	50 ± 2
Hematoporphyrin IX	22 ± 2	40 ± 2
Deuteroporphyrin IX	10 ± 1	20 ± 2
Coproporphyrin III	13 ± 2	29 ± 1
Uroporphyrin III	2 ± 1	5 ± 2
Hemin	2 ± 1	5 ± 2
Bilirubin	3 ± 1	5 ± 2

Guanylate cyclase activity was measured as described under "Materials and Methods". The concentrations studied were 25 μM for the various compounds with the exception of protoporphyrin IX and hematoporphyrin which were at 100 μM. Activity values are expressed as the mean ± SD of triplicate determinations.

III was active. An intact, planar, metal-free porphyrin structure was required for activation, since a metalloprotoporphyrin such as hemin possessed no activating property. Opening of the tetrapyrrole ring, as in bilirubin, rendered the molecule inactive.

In the absence of protoporphyrin IX, the double reciprocal plot of the trypsin solubilized guanylate cyclase activity *versus* Mg^{2+}-GTP concentration exhibited two slopes (Fig. 2) corresponding to two apparent Km values, one of 0.12 mM and the other greater than 2 mM. V_{max} values were 10 and 70 pmol cyclic GMP formed per min per mg of protein for the high and low affinity catalytic sites, respectively. Addition of protoporphyrin IX resulted in a linear plot with a single apparent Km value of 0.12 mM and a V_{max} of 70 pmol/min/mg.

As shown in Fig. 3, the membrane enzyme required 1 and 2 mM excess Mn^{2+} and Mg^{2+}, respectively, for maximal activity. Addition of protoporphy-



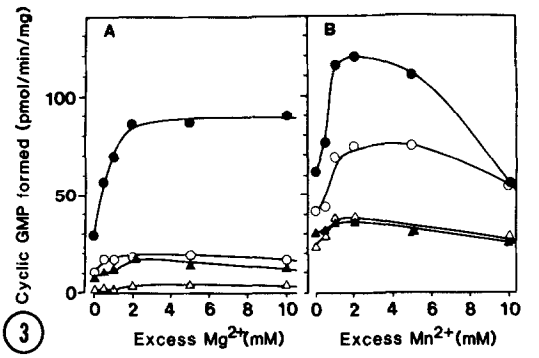


Figure 2: Effect of protoporphyrin IX on the Mg-GTP-dependency of trypsin solubilized guanylate cyclase (double reciprocal plots). The trypsin solubilized guanylate cyclase was incubated at 37°C for 10 min in the absence (○) or in the presence (●) of 10 µM protoporphyrin IX as described under "Materials and Methods".

Figure 3: Effect of excess Mg^{2+} and Mn^{2+} on the protoporphyrin IX activation of membrane-bound and trypsin solubilized guanylate cyclase activities. Trypsin solubilized (○ , ●) and membrane (△ , ▲) fractions were tested for guanylate cyclase activity in the presence of the indicated concentrations of excess Mg^{2+} (A) and Mn^{2+} (B) over 1 mM Me^{2+}-GTP without (○ , △) and with (▲ , ●) 10 µM protoporphyrin IX.

rin IX did not lower the optimal Me^{2+} requirement. The protoporphyrin IX activation observed in the presence of excess Mn^{2+} (+ 60 % at 2 mM) was completely abolished at 10 mM Mn^{2+} although no inhibitory effect due to excess Mg^{2+} was observed.

CONCLUSION

In the presence of Mg^{2+}, protoporphyrin IX activates the membrane-bound guanylate cyclase (5- to 10-fold) to the level of the Mn^{2+}-dependent activity. When measured as a function of Mg-GTP concentration, the trypsin solubilized guanylate cyclase activity exhibits double reciprocal plot with two slopes corresponding to apparent Km values of 0.12 mM and 2 mM. At the present time, we can not distinguish between negative cooperativity and the presence of 2 different classes of catalytic sites for Mg-GTP. Low affinity sites with apparent Km values in the mM range has been reported for the particulate enzyme from various origin (27-29) while lower Km values (100 µM) were reported for the cytosolic enzyme (30, 31). We report here that addition of protoporphyrin IX activated guanylate cyclase by lowering the Mg-GTP requirement and resulted in a linear double reciprocal plot with an apparent

Km of 0.12 mM only. According to Wolin et al (31) the cytosolic enzyme exhibits normal Michaelis-Menten kinetics in the presence of Mg^{2+}, with or without protoporphyrin IX. Activation was reported to result in both an increase in the V_{max} and a slight decrease in the Km (31).

Membrane guanylate cyclase is activated by excess metal, but to a lesser extent than the cytosolic enzyme (20, 32). Furthermore, a lower concentration of metal produced maximal activity (1, 20, 30). Protoporphyrin IX does not alter the excess metal (Mg^{2+} and Mn^{2+}) requirement for the membrane guanylate cyclase. In contrast, lowering of optimal excess Me^{2+} concentration by protoporphyrin IX was reported for the cytosolic enzyme (16, 31). Protoporphyrin IX-induced activation was abolished by 10 mM Mn^{2+}, but no effect was observed at 10 mM Mg^{2+}.

Therefore, protoporphyrin IX regulation appears not to be a specific property of the cytosolic guanylate cyclase. Since it is not clear which form (if not both) of guanylate cyclase modulates the intracellular cyclic GMP level, and since Mg-GTP is thought to be the natural substrate (1), any modulator of the Mg-GTP requirement of the membrane enzyme might be of physiological relevance. Protoporphyrin which occurs naturally in mammalian tissues (at a concentration in the 0.1 to 1 μM range), (33) might play such a role under normal or under the numerous pathological conditions where increased levels of protoporphyrin have been described (23, 33).

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