Evidence for Specific Binding Sites for Guanine Nucleotides in Adipocyte and Hepatocyte Plasma Membranes

A DIFFERENCE IN FATE OF GTP AND GUANOSINE 5'- (β, γ-IMINO)TRIPHOSPHATE

(Received for publication, February 21, 1975)

YORAM SALOMON* AND MARTIN RODBELL

From the Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20014

Binding and degradation of GTP and guanosine 5'- (β, γ-imino)triphosphate (Gpp(NH)p) by plasma membranes from rat liver and fat cells were investigated. Gpp(NH)p is hydrolyzed predominantly by nucleotide pyrophosphohydrolases in the membranes, whereas GTP is hydrolyzed primarily by nucleotide phosphohydrolases. These enzymes are not specific for the guanine nucleotides since co-addition of the analogous adenine nucleotides spares their hydrolysis. Both Gpp(NH)p and GTP are taken up by the membranes at sites which, to the extent that high concentrations of the corresponding adenine nucleotides fail to inhibit uptake, appear to be specific for guanine nucleotides. Gpp(NH)p taken up at these sites remains essentially intact irrespective of the degree of hydrolysis of unbound Gpp(NH)p by nucleotide pyrophosphohydrolases, indicating that the binding site is incapable of degrading Gpp(NH)p. GTP and GDP inhibit competitively the binding of Gpp(NH)p; the binding constants for the three nucleotides are similar (0.1 to 0.4 \( \mu M \)) and are in the same range required for their effects on adenylate cyclase activity. Binding of the nucleotides is inhibited by sulfhydryl agents, suggesting that a sulfhydryl group is involved in the binding process. In contrast to binding of Gpp(NH)p, uptake of GTP is accompanied by substantial hydrolysis, primarily to GDP, under incubation conditions (high [ATP] plus ATP regenerating system) in which [GTP] in the medium remains essentially constant. GDP bound to the membranes is progressively hydrolyzed to 5'-GMP. Thus, GTP and Gpp(NH)p, although binding to the same specific sites, are differentially susceptible to hydrolysis at their terminal phosphates when bound to these sites. These findings are discussed in terms of the markedly different potencies of GTP and Gpp(NH)p as activators of adenylate cyclase systems.

Several studies have shown that guanine nucleotides are involved in the regulation of adenylate cyclase activity in eukaryotic cells (1, 2). GTP has been reported to affect the binding of glucagon (3) and prostaglandin (4) to specific binding sites in hepatic and thyroid cell membranes, respectively. The importance of guanine nucleotides in the activation of adenylate cyclase has been demonstrated by the finding that glucagon and GTP activate the hepatic adenylate cyclase system by a mechanism involving the participation of both ligands acting, respectively, at sites termed receptor and nucleotide regulatory site. Gpp(NH)p, an analog of GTP that is resistant to hydrolysis at its terminal phosphate by nucleotide phosphohydrolases (5), has been found to be a potent activator of all eukaryotic adenylate cyclase systems tested. Gpp(NH)p caused activation in the presence or absence of hormones irrespective of the hormone receptor coupled to the system and even in the absence of a functional receptor (2). In contrast to Gpp(NH)p, GTP fails to cause marked activation of adenylate cyclase systems in the absence of hormones (6) or, in some cases, even in the presence of hormones (7). GTP inhibits the stimulatory effects of Gpp(NH)p in a competitive manner (7-9), indicating that the nucleotides act at a common site. It has been recently shown that GTP and Gpp(NH)p bind to common sites on turkey erythrocyte membranes with similar apparent affinities (9). One possible explanation for the different potencies of GTP and Gpp(NH)p as activators is that GTP, being susceptible to hydrolysis by nonspecific nucleotide phosphohydrolases, is degraded during incubation with membranes to products that are inactive or even inhibitory. GDP has been shown to inhibit, competitively, the actions of Gpp(NH)p on hepatic adenylate cyclase; GDP also inhibits the adrenocorticotropic hormone sensitive adenylate cyclase system in rat adrenal membranes (10). Addition of an ATP-regenerating system abolished the inhibitory effect of GDP. However, even though GTP levels were maintained under

---

* Present address, Department of Biodynamics, the Weizmann Institute of Science, Rehovot, Israel.

1 The abbreviations used are: Gpp(NH)p, guanosine 5'- (β, γ-imino)triphosphate; App(NH)p, adenosine 5'- (β, γ-imino)triphosphate; cyclic AMP, cyclic adenosine 3'-5'-monophosphates.
these conditions, GTP was much less effective than Gpp(NH)p as an activator of adenylate cyclase (10).

A correlation between the binding of Gpp(NH)p and its activation of adenylate cyclase has been shown for hepatic membranes (8) and turkey erythrocyte membranes (9). In this study, we will show that GTP, GDP, and Gpp(NH)p compete for a common site on hepatic plasma membranes at a concentration range identical to that shown for their effects on hepatic adenylate cyclase activity (8). Gpp(NH)p, when bound, is not hydrolyzed at this site. GTP taken up at the binding site appears to be converted to GDP under conditions in which the levels of GTP were maintained constant in the incubation medium. The implications of these findings will be discussed with respect to the fate of GTP at the nucleotide regulatory site.

**EXPERIMENTAL PROCEDURE**

Materials—[8-3H]GTP (5.5 Ci/mmol) and [8-3H]GDP (1.4 Ci/mmol) were from New England Nuclear. [8-3H]GMP (11.1 Ci/mmol) was from Schwarz Mann. [a-32P]Gpp(NH)p (7.8 Ci/mmol), Gpp(NH)p, and App(NH)p were supplied by International Chemical and Nuclear Corp. GTP, GDP, 5'-GMP, cyclic AMP, N-ethylmaleimide, p-hydroxymercuribenzoate, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

**Preparation of Hepatic Plasma Membranes**—Partially purified plasma membranes from rat liver were prepared by modification of the procedure of Neville (11) as previously described (12), and stored in liquid nitrogen. For further use, membranes were taken up in 25 mM Tris-HCl, pH 7.6.

**GTP and Gpp(NH)p-binding Assay**—The reaction mixture, 100 µl, contained 25 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 0.1 mM App(NH)p, 1 mM dithiothreitol, 10 µM cyclic AMP, and 20 nM to 50 nM [8-3H]Gpp(NH)p; 10 to 50 µg of liver plasma membranes were added to start the reaction. Incubations were carried out at 30°C for 5 min; preliminary experiments showed that binding of Gpp(NH)p to the membranes reached equilibrium under these conditions. Incubation was terminated by adding 3 ml of ice-cold 25 mM Tris-HCl, pH 7.6, and filtering the mixture immediately across Millipore filters (AH 2500, 0.45 µ). The filters were washed sequentially three times with 3 ml of the same solution, the entire washing procedure required 15 to 20 s. Filters were dried by heating for 10 min in an oven at 70°C and were placed in counting vials containing 2 ml of 0.7 M LiCl as well as 12 ml of Aquasol (New England Nuclear) for scintillation counting.

**Chromatography**—Nucleotides were separated by chromatography on polyethyleneimine cellulose plates (Merck) using 1 M LiCl as developing solution (1). Samples were loaded in the presence of 0.01 to 0.02 µCi of carrier nucleotides and 0.1 to 0.2 µCi [3H]GPP(NH)p in a volume of 15 to 20 µl. After development, nucleotide containing spots were visualized under an ultraviolet lamp, cut out, and placed in scintillation vials containing 2 ml of 0.7 M MgCl2, 20 mM Tris-HCl, pH 7.5 (13). The vials were shaken for 30 min upon which 0.5 ml of Aquasol (New England Nuclear) was added for counting purposes. In some experiments, 0.5-cm strips were cut out over the entire length of the chromatogram in order to completely screen for degradation products. Extraction and counting procedures were as described above. Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as standard.

**RESULTS**

**Competitive Interactions of Gpp(NH)p, GTP, and GDP with Hepatic Plasma Membranes**—Previous studies have shown that Gpp(NH)p binds to hepatic plasma membranes and stimulates adenylate cyclase activity in these membranes under the same concentration range with an apparent affinity of about 0.1 µM. It was also shown that GTP and GDP inhibit competitively the actions of Gpp(NH)p on adenylate cyclase (8). In this study we have measured the binding of [3H]Gpp(NH)p under adenylate cyclase assay conditions both in the presence and absence of GTP or GDP. As shown in Fig. 1, a competitive relationship was found for the binding of the three nucleotides; the apparent affinities did not differ greatly. Moreover the affinities for binding were nearly identical to the apparent affinities exhibited for the effects of these guanine nucleotides on adenylate cyclase activity. As determined from the ordinate intercept, the number of binding sites for Gpp(NH)p was 50 pmol/mg of membrane protein. Addition of 0.1 mM 5'-GMP had no effect on Gpp(NH)p binding, which is
consistent with earlier studies (8, 10) showing that 5'-GMP does not alter adenylate cyclase activity.

**Fate of Gpp(NH)p during Incubation**—It is thought that the terminal phosphate of Gpp(NH)p is stable to hydrolysis by nucleotide phosphohydrolases (5). The fate of Gpp(NH)p (using \[^{[\alpha-3P]}\text{Gpp(NH)p}\]) was investigated in the absence and presence of 0.1 mM App(NH)p but under otherwise identical incubation conditions used previously for assaying adenylate cyclase activity in hepatic membranes (8). As shown in Fig. 2, essentially all of the labeled Gpp(NH)p (added at 1 µM) was degraded during incubation for 10 min with hepatic membranes. The products were not fully identified but coincided with 5'-GMP and guanosine 3':5'-monophosphate. Labeled material coinciding with GDP could not be detected which is consistent with the resistance of Gpp(NH)p to hydrolysis by nucleotide phosphohydrolases. In the presence of 0.1 mM App(NH)p, 65% of the Gpp(NH)p in the bulk phase remained intact (Fig. 2, lower right panel). The protective effect of App(NH)p suggests that bulk degradation of Gpp(NH)p involves hydrolysis by nucleotide pyrophosphohydrolases that are not specific for guanine nucleotides.

Of the total labeled nucleotide added, 1.3 and 2.0% were taken up at the binding sites for the guanine nucleotides in the absence and presence of App(NH)p, respectively. Note that 80% of the bound labeled material was identified as Gpp(NH)p irrespective of the presence of App(NH)p. This finding indicates that App(NH)p does not bind to this site and that the binding site does not contain nucleotide pyrophosphohydrolase activity. A small percentage of \[^{3P}\]-containing material, as yet unidentified, was also associated with the membranes.

**Binding of GTP**—GTP is rapidly hydrolyzed by nucleotidases present in hepatic plasma membranes; hydrolysis can be eliminated by the addition of 1 mM EDTA, probably as the result of the chelation of divalent cations present in the membranes (3). Binding of \[^{[\text{3H}]\text{GTP}}\] to hepatic membranes was enhanced markedly in the presence of 1 mM EDTA or 1 mM App(NH)p; combination of EDTA and App(NH)p did not cause further increases in binding (Fig. 3). Thus, as shown above for the effects of App(NH)p on Gpp(NH)p binding, high concentration of App(NH)p failed to inhibit the binding of GTP to membranes. These findings provide further evidence for the high specificity of the binding sites for guanine nucleotides. The nature of the bound labeled nucleotide is discussed below.

**Fate of GTP, GDP, and GMP under Cyclase Assay Conditions**—Preliminary experiments showed that addition of ATP or App(NH)p to the medium used for binding assays spared the hydrolysis of GTP by hepatic membranes (data not shown). The same sparing effects of ATP were observed with purified plasma membranes from rat adipocytes (Fig. 4, A and B). Addition of a nucleotide regenerating system (creatine phosphate, creatine kinase) also served to maintain the levels of GTP when incubated with fat cell membranes. Furthermore, as shown in Fig. 4C, quantitative conversion of GDP to GTP occurred in the presence of the regenerating system. Some conversion of GDP to GTP also occurred in the absence of the regenerating system in the presence of 1 mM ATP. This could be explained by nucleotide phosphotransferase activity in the membranes.

![Fig. 2. Degradation of Gpp(NH)p under adenylate cyclase conditions.](image)

![Fig. 3. Binding of GTP to liver plasma membranes under various conditions.](image)
The chromographic system used in this study did not permit complete separation of 5'-GMP and guanosine. For this reason, the degradation of 5'-GMP by fat cell membranes was not assessed. The only interesting point, illustrated in Fig. 4D, is that 5'-GMP was converted to some extent to GDP and GTP in the presence of ATP and the regenerating system. The dependence on the regenerating system for this conversion raises the possibility that the regenerating system also contains impurities of myokinase-like enzymes and thus, in combination with creatine kinase plus phosphotransferase activity associated with the membranes, could account for these observations. The reported stimulatory effects of GDP and GMP on adenylate cyclase activity studied in several laboratories (7, 15) were carried out in the presence of ATP regenerating systems that can convert GDP to GTP. It is obvious, therefore, that these effects of GDP cannot be simply interpreted. If myokinase is combined with the ATP-regenerating system, then to the extent that this combination converts to GMP to GTP, GMP will also give stimulatory effects (15). Adrenal adenocorticotropic hormone-sensitive adenylate cyclase is stimulated by 10 μM GMP in the presence of ATP and an ATP-regenerating system but not with App(NH)p as substrate and in the absence of regenerating system. The stimulatory effect of GMP is easily explained if it is assumed that 2% of the GMP was converted to GTP (10).

It should be noted also that high concentrations of creatine kinase often used in adenylate cyclase assays may complicate interpretation of the binding and actions of nucleotides. For example, 50 units/ml of creatine kinase (a frequently used concentration) is equivalent to 5 μM enzyme. At this concentration significant binding of nucleotides to the enzyme may occur, particularly if this concentration exceeds the concentration of nucleotide in question. In addition, contamination of the enzyme with other enzymes involved in nucleotide metabolism may be revealed.

**Fate of GTP in Its Free and Bound Forms**—Having established conditions that prevent the bulk hydrolysis of GTP by nonspecific nucleotidases in liver and fat cell membranes, we then investigated the distribution and fate of GTP taken up by these membranes. To our surprise, under conditions in which the levels of GTP in the medium remained nearly constant (80 to 100%) during incubation with the membranes, the bulk of the radioactivity added as GTP that was taken up by the membranes was in the form of GDP (about 66%), and only 25% was identified as GTP; the remainder was in the form of GMP or possibly guanosine. This finding was identical with either fat cell or liver membranes (Fig. 5).

A question of paramount importance was whether this result reflected the hydrolysis of GTP subsequent to binding and isolation of the membranes. It does not seem to reflect uptake of GDP from the medium since, as noted above, GDP and GTP have similar affinities for the binding sites and very little bulk hydrolysis of GTP to GDP occurred under the conditions used in these experiments. It seems more likely that GTP taken up at the specific binding sites was hydrolyzed to GDP and that the distribution of labeled nucleotides observed in the washed membranes reflects hydrolysis of GTP at or near the binding sites. Interestingly, when the washed membranes on the filters were allowed to stand for 3 to 5 min prior to treatment with acetone (the means used to stop further reactions), slow,
Effects of sulfhydryl reagents on binding of GTP to hepatic plasma membranes

Inhibition of GTP binding by N-ethylmaleimide (NEM) and p-mercuribenzoate (PMB). Binding of [8-3H]GTP was determined as described in the legend to Fig. 3 and under "Experimental Procedure." The concentration of liver membrane protein was 18 µg/100 µl of assay medium; the incubation time was 5 min at 30°C.

| GTP concentration | GTP bound |
|-------------------|-----------|
| µM                | pmol/mg/protein |
| 0.07              | 25        |
| 0.2               | 46        |
| 2.0               | 69        |

Effects of Sulfhydryl Agents on GTP Binding—It was of interest to determine the effects of sulfhydryl agents on the binding of guanine nucleotides to hepatic and fat cell membranes since it has been reported that sulfhydryl agents such as p-mercuribenzoate and N-ethylmaleimide inhibit adenylate cyclase activity in these membranes (12). Table I shows that both p-mercuribenzoate (10 µM) and N-ethylmaleimide (1 mM) inhibit binding of GTP to hepatic plasma membranes; dithiothreitol completely prevented the inhibitory effects of p-mercuribenzoate. Similar findings were obtained with fat cells (data not shown). Inhibition by the sulfhydryl agents seems not to be of a strict competitive type and was not prevented even with high concentrations of GTP. It remains to be determined whether the sulfhydryl agents also affect interaction of ATP at the catalytic site of adenylate cyclase.

DISCUSSION

We have shown in this study that liver and fat cell plasma membranes contain sites which, to the extent that ATP (or App(NH)p) fails to compete, seem highly specific for guanine nucleotides. GTP, GDP, and Gpp(NH)p interact competitively with these binding sites with affinities in the range of 0.1 to 0.4 µM. This concentration range is identical with that found for the actions of these nucleotides on the hepatic adenylate cyclase system (8). Such findings do not, of course, establish that all of the binding sites are necessarily identical with the sites through which the nucleotides alter adenylate cyclase activity. We have shown earlier (8) that the binding and actions of Gpp(NH)p are linked phenomena suggesting that at least part of the binding sites are related to the so-called nucleotide regulatory site.

The plasma membranes of hepatic and fat cells clearly contain enzymes that degrade GTP and Gpp(NH)p, and GDP. In contrast to the highly specific binding sites for the guanine nucleotides, degradation of these nucleotides was effectively inhibited by ATP or App(NH)p. Addition of the adenine nucleotides did not inhibit binding of the guanine nucleotides at their specific binding sites. We conclude, therefore, that the binding sites for the guanine nucleotides do not represent the nucleotidases in the membranes responsible for the bulk, non-specific hydrolysis of these nucleotides. App(NH)p actually increased the binding of GTP and Gpp(NH)p, presumably as a result of the protection against hydrolysis by the nucleotidases.

Gpp(NH)p was taken up at the specific binding sites irrespective of the degree of hydrolysis by non-specific nucleotide pyrophosphohydrolases (Fig. 2) and when bound to the membranes retained its structure. Clearly, the binding site does not have the capacity to degrade Gpp(HP)p, i.e. it does not contain pyrophosphohydrolase activity.

When GTP binding was measured in the presence of 1 mM ATP and 1 mM EDTA, conditions which reduce bulk hydrolysis of GTP by non-specific nucleotidases, GTP taken up at the specific binding sites was primarily in the form of GDP. Since GDP does not have a greater affinity for the binding sites than GTP, and therefore should not be selectively taken up from the medium, such findings suggest that the bound GDP was formed from GTP initially taken up at the binding sites. GDP-GMP, which has negligible affinity for the binding sites, was formed at the expense of GTP and GDP (cf. Fig. 5). For reasons discussed earlier, it is difficult to establish the original ratio of GTP, GDP, and GMP in the bound form; nor are data at hand to identify the site or sites of degradation with the observed binding sites. Nevertheless, it appears that though GTP and Gpp(NH)p bind competitively at the same binding sites and with high specificity, GTP is susceptible to hydrolysis at or near the sites with resultant formation of GDP and GMP.

In view of these findings, the possibility can be raised that the stability of GTP and Gpp(NH)p at the binding sites can be correlated with the efficacy of their actions on adenylate cyclase activity. If sequential degradation of GTP to GDP at the nucleotide regulatory site is plausible, this would serve as a reasonable mechanism for clearing GTP from the regulatory site. Obviously, such a clearing mechanism does not apply to Gpp(NH)p and, in fact, Gpp(NH)p has been shown to remain strongly bound to binding sites on the hepatic membranes even after extensive washing of the membranes (8).

Obviously, further investigation with purified preparations of adenylate cyclase response to the actions of guanine nucleotides will be required to establish whether GTP is hydrolyzed during the process of activation of the enzyme. We are tempted to speculate based on the findings presented here that such a mechanism could play an important role in the regulation of these complex enzyme systems. Hydrolysis of GTP at the regulatory site would demand a constant supply of GTP for maintenance of enzyme activity. Hormones may control this process by increasing the formation of states that have higher affinities for GTP or lower hydrolysis of the nucleotide, or both. It has been shown that glucagon increases the rate of isomerization of Gpp(NH)p-ligated states of the hepatic enzyme to more active states of the enzyme (8) probably because the high activity states have a higher affinity for Gpp(NH)p (10).

REFERENCES

1. Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. (1971) J. Biol. Chem. 246, 1877-1892
2. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J., and Rodbell, M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3087-3090
3. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1877-1878
4. Moore, Wayne, V., and Wolff, J. (1973) *J. Biol. Chem.* 248, 5705-5711
5. Eckstein, F., Kettler, M., and Parmeggiani, A. (1971) *Biochem. Biophys. Res. Commun.* 45, 1151-1158
6. Rodbell, M., Lin, M. C., and Salomon, Y. (1974) *J. Biol. Chem.* 249, 59-65
7. Lefkowitz, R. J. (1974) *J. Biol. Chem.* 249, 6119-6124
8. Salomon, Y., Lin, M. C., Londos, C., Rendell, M., and Rodbell, M. (1975) *J. Biol. Chem.* 250, 4239-4245
9. Spiegel, A. M., and Aurbach, G. D. (1974) *J. Biol. Chem.* 249, 7630-7636
10. Londos, C., and Rodbell, M. (1975) *J. Biol. Chem.* 250, 3459-3465
11. Neville, D. R. (1976) *Biochim. Biophys. Acta* 154, 540-552
12. Pohl, S., Birnbaumer, L., and Rodbell, M. (1971) *J. Biol. Chem.* 246, 1849–1856
13. Ramderath, K., and Ramderath, E. (1967) *Methods Enzymol.* 12, 323–347
14. Lowry, D. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
15. Sato, S., Yamada, T., Furuta, P., and Makinchi, M. (1974) *Biochim. Biophys. Acta* 332, 166–174
16. Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., and Berman, M. (1975) in *Advances in Cyclic Nucleotide Research* (Drummond, G. I., Greengard, P., and Robison, G. A., eds) Vol. 5, Raven Press, New York
Evidence for specific binding sites for guanine nucleotides in adipocyte and hepatocyte plasma membranes. A difference in fate of GTP and guanosine 5′-(beta, gamma-imino) triphosphate.
Y Salomon and M Rodbell

*J. Biol. Chem.* 1975, 250:7245-7250.

Access the most updated version of this article at [http://www.jbc.org/content/250/18/7245](http://www.jbc.org/content/250/18/7245)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/250/18/7245.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/250/18/7245.full.html#ref-list-1](http://www.jbc.org/content/250/18/7245.full.html#ref-list-1)