Advances in Cyclic Nucleotide Research

Volume 5

Second International Conference on Cyclic AMP
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EDITORS:

George I. Drummond
Professor of Biochemistry
University of Calgary
Calgary, Alberta, Canada

Paul Greengard
Professor of Pharmacology
Yale University School of Medicine
New Haven, Connecticut

G. Alan Robison
Director, Program of Pharmacology
University of Texas Medical School
Houston, Texas

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Preface

This volume of *Advances in Cyclic Nucleotide Research* contains most of the invited lectures presented at the Second International Conference on Cyclic AMP held in Vancouver, Canada, on July 8–11, 1974. Abstracts of papers presented by other investigators are also included. The latter were all presented by the poster technique. To speed publication of the volume, manuscripts have received minimal editorial modification and are presented largely in the form chosen by the authors.

Research in the area of cyclic nucleotides continues to expand and accelerate. We hope this volume, which deals with many, but not all, aspects of cyclic nucleotide metabolism and function, will stimulate further productive research and provide deeper insights into the role of these compounds in cellular regulatory processes. The Vancouver conference, we hope, also accomplished these objectives.

George I. Drummond
Paul Greengard
G. Alan Robison

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Opening Remarks

T. W. Rall

Case Western Reserve University, Cleveland, Ohio

It is indeed a privilege to participate in this the Second International Conference on Cyclic AMP. I would like to express, on behalf of the participants and guests, appreciation to the University of British Columbia for its sponsorship. In addition, special recognition should be given to the principal architect of the Conference, Dr. George Drummond, for his extraordinary efforts in all phases of its organization.

I am sure all participants recognize and understand the pioneering contributions of Dr. Earl W. Sutherland which form the foundation for this entire Conference and are saddened by his premature death just a few months ago. The literature dispassionately documents his work that led to the discovery of cyclic AMP itself and to the appreciation of the widespread importance of cyclic AMP as a regulatory molecule. But the literature cannot adequately portray the power of his intuitive approach, unfettered by existing dogma yet disciplined by careful experimental observation and cautious interpretation. Neither can it portray his concern for the environment of scientific activities that was expressed by his encouragement of the independence of younger collaborators and of open communication among workers in the field. While we will miss Earl Sutherland, his legacy of discoveries and ideas should help us answer many of the questions that will be posed in this Conference.

I have always been impressed by the fact that Dr. Sutherland doggedly focused on the regulation of glycogen phosphorylase in his investigation of the mechanism of hormone-induced glycogenolysis despite the then extant belief that phosphorylase was responsible for both the synthesis and degradation of cellular glycogen. He was never convinced that because phosphorylase can be observed to synthesize glycogen at a rate faster than the reverse reaction it negated the observation that hormone-induced glycogen breakdown was associated with augmented levels of recoverable phosphorylase activity. By the same token I believe Dr. Sutherland would be dismayed if we or future workers were to coagulate his enormously useful second-messenger concept into immutable dogma. Obviously, observations that a given hormone can elevate cellular levels of cyclic AMP or that exogenous cyclic AMP can mimic the hormone's effects do not permit the conclusion that the only consequence of hormone-receptor interaction is alteration of adenylate cyclase activity. Neither do these observations permit the conclusion that changes in cellular cyclic AMP levels are sufficient to explain altered cell function under physiologic conditions, espe-

cially in those circumstances where it is difficult to detect alterations in cyclic AMP metabolism (e.g., low hormone concentrations). Undoubtedly these or related issues will be raised during this Conference, especially by Drs. Goldberg and Rasmussen.

Some of the questions before us here, such as the role of cyclic AMP in the regulation of cell proliferation and in the expression of malignant transformation, are relatively new ones and were not discussed at the previous Conference. Others, such as the mechanism of hormonal activation of adenylate cyclase, have been with us since the beginning, with only modest advances in knowledge despite more than 15 years of effort by a number of laboratories. Fortunately, this challenging and important topic with its ramifications into general questions concerning structure and function of cell membranes continues to attract the attention of skilled investigators. Still other questions, such as the mechanism of action of cyclic AMP, have also been with us since the beginning and have been advancing steadily through the years.

Attention now is focused on cyclic AMP-"dependent" protein kinases and their substrates, at least in animal cells, even though only in the case of the regulation of glycogen metabolism has it been possible to relate phosphorylation to altered functional properties of proteins. As we hear more about membrane-bound protein kinases, the "autophosphorylation" of regulatory subunits, and the impact of "substrate" and "nonsubstrate" proteins on the properties of protein kinases in the days ahead, a picture of pre-existing multimolecular complexes may begin to emerge. Either these complexes are part of a membrane matrix whose properties might be changed by combination with cyclic AMP with or without protein phosphorylation, or these complexes may become associated with other cellular components as a consequence of interaction with cyclic AMP, again with or without phosphorylation of the "initial" or "final" complexes. In any event some conceptualization must emerge to replace the unsatisfying picture of the catalytic subunit of protein kinase swimming about, happily phosphorylating a variety of cellular constituents whether they need it or not.

Over the years, I have found working with cyclic AMP a most interesting and exciting experience. It has provided me with a broad and continuing postgraduate education in the biologic sciences and brought me in contact with people and questions in an array of disciplines ranging from molecular biology to neurophysiology. This Conference and this volume are no exception. Perhaps we should feel uncomfortable, all crowded under the umbrella of cyclic AMP, and perhaps we should be concerned lest we become unduly mechanistically doctrinaire by focusing attention on cyclic AMP. On the other hand, if we remember that the discovery of cyclic AMP was a product of an investigation into the mechanism of cellular regulation by a hormone and that the search for understanding the regulation of tissue and cell function is the real bond that unites us, then I am confident of a productive outcome.

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Role of Adenine and Guanine Nucleotides in the Activity and Response of Adenylate Cyclase Systems to Hormones: Evidence for Multisite Transition States

Martin Rodbell, Michael C. Lin, Yoram Salomon, Constantine Londos, James P. Harwood, Bruce R. Martin, Marc Rendell, and Mones Berman

Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases; and Laboratory of Theoretical Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

The adenylate cyclase system is a key control point in the actions of biogenic amines, prostaglandins, and a number of peptide hormones. This system is an intrinsic component of the cell membrane in eucaryotic cells and is thought to consist of distinct receptor and catalytic units that are somehow articulated within the membrane; binding of the effectors to the receptors engenders changes in the activity of the catalytic units (Sutherland, 1972). Little is known of the molecular architecture of the system. Investigations of these systems have centered principally on the binding of the effectors to target cell membranes containing adenylate cyclase and on the kinetics of cyclic AMP formation from the enzyme's substrate, ATP, in response to the effector molecules.

In developing a model for hormone action on adenylate cyclase, our studies concentrated on the properties of the glucagon-sensitive adenylate cyclase system in rat liver plasma membranes (Rodbell, 1972). These studies revealed that the enzyme system is far more complex than previously realized, since the enzyme system contains at least three sites through which ligands interact and affect enzyme activity: the hormone receptor site; the nucleotide site, which reacts preferentially with GTP but which also appears to react with high concentrations of ATP; and the catalytic site, which reacts with $MgATP^{2-}$, the productive form of substrate. The nucleotide regulatory site was discovered first with studies of the hepatic system, which showed that GTP (or ATP) increased the rate of dissociation of glucagon from its receptor site and that hormone-stimulated activity was dependent on the presence of GTP when the ATP concentration was reduced. It became obvious that investigations of the binding and actions of glucagon were in-

sufficient to provide an adequate understanding of the activation process; the actions of GTP must also be understood.

Any model must also take into account the binding and catalysis of ATP at the catalytic site and the role that magnesium ion, which is required for catalysis, plays in this process. While it is generally agreed that the productive substrate is $MgATP^{2-}$, magnesium ion has other effects on adenylate cyclase systems that cannot be accounted for by its chelation with ATP at the active site (Perkins, 1973). Magnesium ion concentrations in moderate excess of substrate result in enhanced enzyme activity in several systems. In addition to this effect, excess magnesium ion causes marked reduction in stimulation of the hepatic system by glucagon (Pohl, Birnbaumer, and Rodbell, 1971). These effects also need to be evaluated.

The nature and kinetics of the molecular transitions that occur on binding of the hormone and GTP at their respective sites on the enzyme system must be evaluated. With soluble, highly purified regulatory enzymes, molecular transitions occurring in response to ligands can be visualized with a variety of chemical and physical probes. However, for a membrane-bound enzyme such as adenylate cyclase, which represents but a fraction of the total protein in the membrane, such probes at best give ambiguous data. At present the kinetic characteristics of cyclic AMP formation are our only useful means of measuring a functional change in the enzyme as it undergoes molecular transitions. Particularly useful information can be obtained if cyclic AMP accumulation displays time-dependent (i.e., transient) kinetics subsequent to the addition of hormones and activating nucleotide. As discussed by Frieden (1970) and Ainslee, Shill, and Neet (1972), transient kinetics may indicate that the enzyme exists in different transitional states which have different kinetic properties at the active site, and that the equilibrium between these states is slow. Ligand binding may shift the equilibrium between these states, which is reflected by a change in activity and in cooperative responses. In addition to conformational changes, cooperative kinetics may reflect rearrangement of substrate at the active site, association-dissociation reactions between subunits, and a change in binding of other ligands that may constrain or influence the activity of the enzyme. Studies of transient kinetics of adenylate cyclase require a precise and sensitive assay procedure, which we developed (Salomon, Londos, and Rodbell, 1974).

Studies of the transient kinetic behavior of adenylate cyclase are described here. The actions of guanine nucleotides were investigated with the use of Gpp(NH)p, an analogue of GTP that is not hydrolyzed by nucleotide phosphohydrolases and which has proved particularly valuable for evaluating the role of the nucleotide regulatory site in the activation process (Londos, Salomon, Lin, Harwood, Schramm, Wolff, and Rodbell, 1974). App(NH)p, a substrate for adenylate cyclase that also is not hydrolyzed by nucleotide phosphohydrolases in plasma membranes (Rodbell, Birnbaumer, Pohl, and Krans, 1971), was used to evaluate the kinetic characteristics of substrate interaction and catalysis at the active site.

We first demonstrated qualitatively that hepatic adenylate cyclase exists in different states of activity governed by the liganding of glucagon at the receptor site; Gpp(NH)p at the regulatory site; and protonated substrate ($HApp(NH)p^{3-}$), a potent inhibitor, and $MgApp(NH)p^{2-}$ at the active site. These findings indicated that the system was too complex to be explained by a simple mechanism and that a more formal mathematical modeling approach would be useful. Based on the nature of the data and accepted concepts in enzyme kinetics, several multistate models were formulated and tested (Rendell, Salomon, Lin, Rodbell, and Berman, *submitted*). Least-square-data-fitting criteria were employed to test for uniqueness and consistency of the models (Berman and Weiss, 1967). These attempts led to an acceptable model with the assumptions that the variables involved in catalysis—V_{max}, K_m for $MgApp(NH)p^{2-}$, and K_i for $HApp(NH)p^{3-}$—of substrate are common to all states of the enzyme. It was inferred from the model that differences in catalytic activity are due to changes in the kinetic constants related to the variables. It was also concluded that there are at least three identifiable enzyme states and that transitions between these states are determined by the activating ligands. The model brought out certain inadequacies of the data, which led to the design of new experiments that permitted better definition of the system. The model was able to explain quantitatively the observations made with both the hepatic and adrenal adenylate cyclase systems, and provided a qualitative explanation for the complex actions of guanine nucleotides on the multireceptor fat cell adenylate cyclase system.

The implications of the model are discussed in terms of its general application to hormone-activated adenylate cyclase systems, its possible physiologic significance, and for future studies of the molecular basis of hormone- and nucleotide-induced activation of these systems.

EFFECTS OF GTP ON GLUCAGON BINDING AND ACTION

It is generally agreed that activation of adenylate cyclase by hormones involves an intermediate process—transduction—between the process of the hormone binding to its receptor and the presumed conformational change in the catalytic unit, which results in increased enzymatic activity. The precise nature of the transduction process and indeed the molecular basis of hormone action remain unknown. Studies of the effects of GTP on the binding and action of glucagon on the hepatic adenylate cyclase system provided some insight into the nature of the transduction process (Rodbell, 1972). Briefly, it was found that glucagon binding to specific sites in these membranes was changed from a slowly reversible to a rapidly reversible process by adding GTP in concentrations as low as 10^{-8} M; this effect of GTP was mimicked by concentrations of ATP, the substrate of adenylate cyclase, at least three orders of magnitude higher than that required for GTP. Since GTP is not a substrate for adenylate cyclase and since the

nucleotide does not share any structural feature in common with glucagon, it was reasoned that the effects of GTP and ATP on binding were due to their action at a site independent of the receptor and active sites. Since ATP acts as substrate and also affects hormone binding, the role of GTP in the expression of glucagon-stimulated activity of the enzyme could not be evaluated unless the ATP concentration was reduced to levels that did not alter hormone binding. When this was done, it was found that the stimulatory effects of glucagon were reduced markedly. Under these conditions GTP addition caused marked stimulation of enzyme activity in the presence of the hormones. This effect of GTP was observed also in the presence of App(NH)p, a substrate which unlike ATP is not hydrolyzed by nucleotide phosphohydrolases in the membranes; this finding precluded the possibility that the stimulatory effect of GTP was due simply to the sparing of ATP hydrolysis by these enzymes.

The findings that GTP increased the rate of dissociation of glucagon from its specific binding sites and also enhanced activation of adenylate cyclase by the hormone seemed paradoxical. The possibilities were raised that the binding sites are not related to the true receptor for glucagon and/or that the effects of GTP on binding and action of glucagon are not related (Birnbaumer and Pohl, 1973). However, recent studies showed that occupation of all the specific binding sites for glucagon is required for full activation of the hepatic adenylate cyclase system by glucagon, and that GTP by stimulating the release of glucagon from its receptor causes decay in glucagon-stimulated activity when the only source of hormone is that bound to the receptors (Rodbell, Lin, and Salomon, 1974). In a previous study (Rodbell et al., 1974) a complex relationship was found between receptor occupation and enzyme activity as a function of the concentration of activating nucleotide. For example, it was found that approximately 50% occupation of the receptors was required to obtain half-maximal activation by glucagon in the presence of 0.1 mM App(NH)p as substrate and source of activating nucleotide. This was estimated from the finding that half-maximal occupation of receptors occurs with 5 nM glucagon (Rodbell et al., 1971), which is the concentration giving half-maximal activation of the enzyme (Fig. 1). By contrast, addition of GTP shifted the concentration of glucagon required for half-maximal activation down to 0.5 nM; from direct measurements of glucagon binding, it was estimated that about 10% occupation of the receptors yielded about 60% activation of the enzyme in the presence of GTP at its maximally effective concentration (1 μM). However, full occupation of the receptors was required for maximal activation in the absence or presence of GTP. Thus the relationship between enzyme activity and hormone concentration (or receptor occupation) does not reflect simply the level of receptors occupied by the hormone; a critical factor in this relationship is the concentration of GTP (or ATP) and the actions of the nucleotide at the nucleotide regulatory site. These findings provided strong evidence that activa-

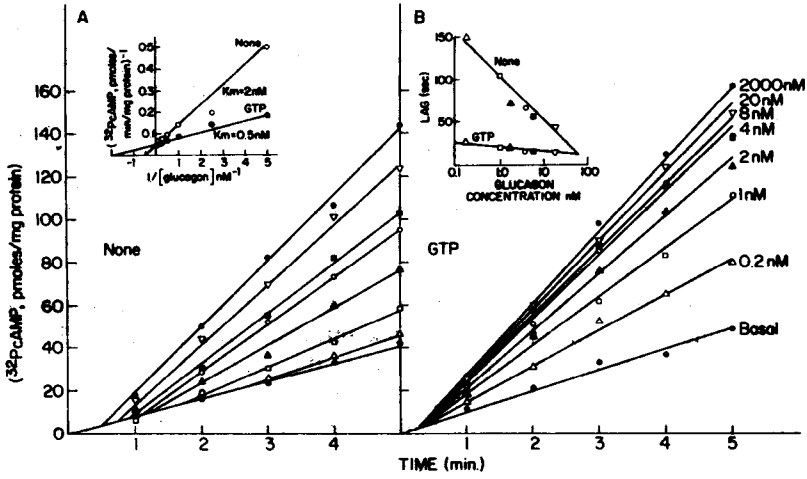

FIG. 1. Time course of glucagon action on hepatic adenylate cyclase in the absence (*A*) and presence (*B*) of 1 μM GTP. Glucagon concentrations are indicated in *A*. *Inset* in *A* contains data plotted from steady-state rates. (From Rodbell, et al., 1974)

tion of the adenylate cyclase system in hepatic membranes involves the participation of two ligands—GTP (or ATP) and hormone—acting in concert. The complex relationship between receptor occupation and nucleotide concentration on activity could be explained if glucagon acts by facilitating activation of the enzyme by the nucleotides. In other terms, the transducer function could involve structural changes in the enzyme that facilitate nucleotide activation at the regulatory site either by increasing the affinity of this site for the nucleotides or by increasing the rate of transformation of the nucleotide-occupied enzyme to the activated state. According to this concept, glucagon serves to regulate activation by the nucleotide rather than serving as an activator *per se*.

Figure 1*A* shows that in the absence of added GTP glucagon stimulates adenylate cyclase activity with lags in onset that are inversely related to the concentration of glucagon (Fig. 1*B*, *inset*). The lag phase was not linearly correlated with the rates at which glucagon binding to its receptor reached equilibrium, which suggested that the lag was not due solely to this process. In Fig. 1*B* it is seen that GTP (1 μM) essentially eliminated the lag phase at all hormone concentrations. Moreover, addition of the nucleotide decreased the concentration of glucagon required for half-maximal stimulation of enzyme activity (Fig. 1*A* *inset* and 1*B*). The cooperative kinetics displayed with the two ligands suggested that the enzyme system exists in different transition states, the equilibrium between these states being influenced by the binding of glucagon to its receptor and GTP to the nucleotide regulatory site. Pretreatment of the enzyme system with glucagon followed by exten-

sive washing of the membranes to remove all but bound glucagon resulted in the loss of the lag phase at all concentrations of glucagon tested.

The question at this point was whether GTP further increases the activity after pretreatment with glucagon and washing the membranes. In the experiments described in Fig. 2, ^{125}I-glucagon was added during pretreatment to follow the fate of the bound hormone during subsequent incubation under adenylate cyclase conditions. The pretreated enzyme system displayed an immediate increase in activity over control, untreated membranes (basal activity); labeled hormone did not dissociate from the receptor sites in the absence of guanine nucleotide. The resultant activity proved marginal, however, since addition of GTP at concentrations ranging from 30 nM to 1 μM resulted in increased rates of activity; the maximal rate was achieved with 1 μM GTP. Note that although 0.1 mM GTP did not exert further effects on enzyme activity, the nucleotide at this concentration caused further increases in the rate at which glucagon dissociated from its receptor, indicating that this dissociation is not causally related to the increased activity seen with GTP but probably reflects a change in the kinetics of hormone binding to the GTP-activated state of the enzyme. The implication of this finding is that the hormone receptor site has been changed to another configuration or structure when the enzyme is in its activated state; the active state, according to these findings, is the state displaying rapid dissociation of the hormone. Occupation of the hormone-induced state by GTP and resultant conversion to the activated state results in rapid turnover of glucagon at the receptor site, a phenomenon that ensures dynamic control of glucagon action on the system. In this regard one of the interesting outgrowths of this experiment was the finding that the activated state of the enzyme induced by GTP and hormone maintained a constant rate of activity for about 2 min

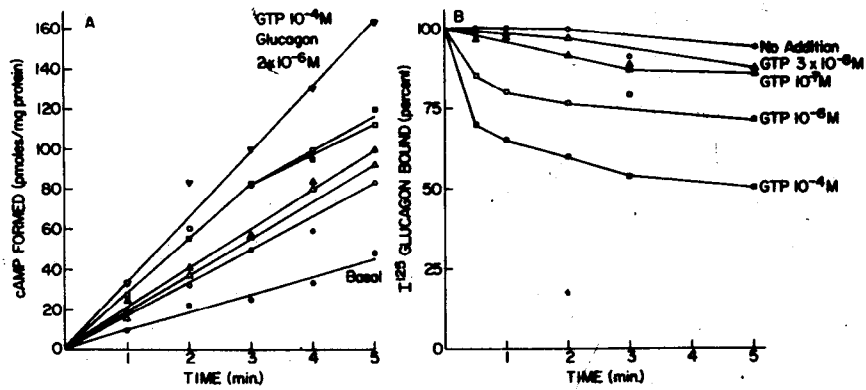

FIG. 2. Effects of GTP on hepatic membranes pretreated with ^{125}I-glucagon. Concentrations of GTP added to adenylate cyclase medium (*A*) and to medium used for binding studies (*B*) are indicated in *B*. (From Rodbell et al., 1974)

during rapid dissociation of the hormone; thereafter the rate declined to steady-state levels slightly above that of basal activity. A possible explanation for the relatively slow rate of decay of the GTP-activated state after dissociation of glucagon is provided by studies with Gpp(NH)p, a synthetic analogue of GTP.

ACTIVATION OF ADENYLATE CYCLASE BY GUANYLYLIMIDODIPHOSPHATE

Hepatic System

Evidence for Transition States

Previous studies showed that Gpp(CH_2)p, a synthetic analogue of GTP that is resistant to hydrolysis by nucleotide phosphohydrolases, mimics the stimulatory effects of GTP on the hepatic adenylate cyclase system (Rodbell et al., 1971). Gpp(NH)p is also resistant to hydrolysis by these enzymes but, in contrast to GTP or Gpp(CH_2)p, causes marked activation of adenylate cyclase in the absence of glucagon (Fig. 3). Both synthetic guanine nucleotides display lags in onset of activation. Addition of glucagon (2 nM) diminished the lag phase of Gpp(NH)p and Gpp(CH_2)p activation and increased the rates observed compared to those seen in the absence of the hormone (Fig. 3). Most importantly, this effect of glucagon on the lag phase was seen during the time period in which the hormone alone exerted no

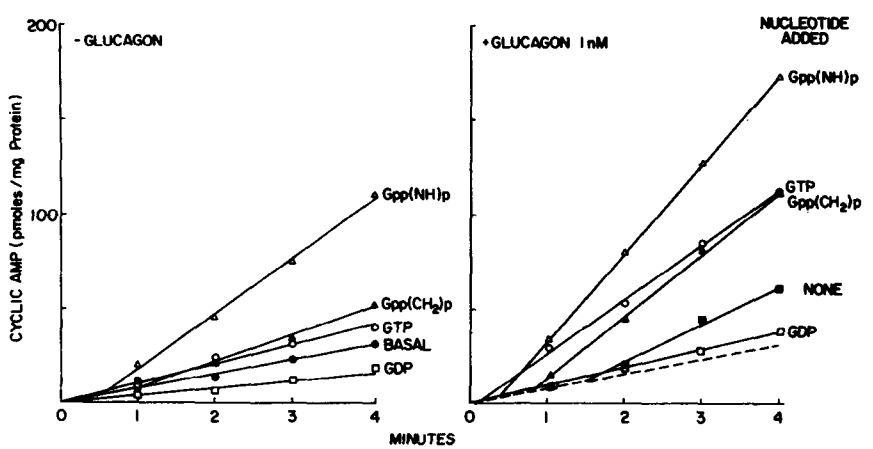

FIG. 3. Effects of several guanine nucleotides on hepatic adenylate cyclase activity in presence and absence of glucagon. The assay medium contained 25 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM α-^{32}P-App(NH)p, and 1 mM cyclic AMP. Guanine nucleotides were tested at 0.1 mM. Incubation temperature was 30°C. (From Salomon et al, *submitted*)