Ann. Rev. Pharmacol. Toxicol. 1978. 18:431–59

COMPARTMENTALIZATION OF CYCLIC NUCLEOTIDE–MEDIATED HORMONE ACTION

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INTRODUCTION

This review synthesizes the recently published information on the role of cyclic nucleotides in hormonal action. Our thesis is that hormonal regulation of cyclic nucleotide levels and the subsequent molecular action of cAMP and cGMP are localized intracellular phenomena and that the eventual understanding of hormone action will depend upon knowledge of how effector molecules alter biochemical processes at precise intracellular loci. Studies that document compartmentalization of cyclic nucleotides within the cell are presented and the discrepancies in the literature that might be explained by this concept are reviewed. Our overall approach is mechanistic and we do not attempt to catalog the many important publications which have increased the organ- or tissue-specific knowledge of cyclic nucleotide metabolism. Rather, we emphasize the findings that appear to us to have wide applicability in the understanding of the regulation of cyclic nucleotide localization, tissue level, and action. Possible interrelationships between the biochemical effects of cAMP and cGMP as well as Ca^{2+} are considered.

During the past five years important advances have been made in understanding the molecular mechanism by which cyclic nucleotide levels and action are regulated. The distinct metabolic components of the cyclic nucleotide systems have been separated from the whole cell, purified to varying degrees, and studied in vitro with increasing biochemical sophistication. Several complex regulatory determinants for the adenylate and guanylate cyclases have been proposed and successfully tested experimentally. Great strides have been made in solidifying the concept of cell surface receptors for a variety of hormones and neurotransmitters. Knowledge of the type and specificity of the phosphodiesterases has been extended. The properties of cAMP-dependent protein kinases are now well detailed and several natural substrates have been isolated. Progress in the field of cGMP-dependent protein

0362-1642/78/0415-0431$01.00

kinase has also been made. Lastly, several totally new regulatory elements have been described that result in autoregulation of cyclic nucleotide tissue levels or that allow integration of other intracellular effectors with the cyclic nucleotide systems.

And yet, in spite of the elegant work on the molecular detail of cyclic nucleotide metabolism, many of the central questions of interest remain. In particular, investigation into the control of steroidogenesis by hormones and the control of glycogenolysis by α-adrenergic stimuli have raised doubts about the role of cAMP as a second messenger. Most recently, several groups have applied novel technology to the study of the two above-mentioned processes. This and data from other investigators have demonstrated the importance of compartmentalization of cAMP into active intracellular pools, as well as the importance of alternative second messengers such as Ca^{2+}. The relationship between cGMP and hormones has been even more difficult to assess. The lack of direct hormonal effects on guanylate cyclase and the paucity of knowledge regarding cGMP regulation of biochemical processes have compounded this conceptual void. In this review we compare and contrast the literature on cyclases, phosphodiesterases, and protein kinases for cAMP and cGMP in an attempt to demonstrate how hormones affect the level and action of these two messengers. We conclude with a speculative section on techniques that can be used to study compartmentalization of cAMP and cGMP. Hopefully, this approach will add to the knowledge of how extracellular stimuli control intracellular processes.

ADENYLATE CYCLASE

The knowledge of the molecular aspects of how hormonal binding to the plasma membrane affects adenylate cyclase activity, while not complete, has advanced to the stage where the system can be used as a model to study plasma membrane function. The use of newer techniques such as eucaryote mutant cell lines (1), cell-cell fusion (2), and biophysical membrane analysis (3) in addition to more classic modes of investigation have made this one of the most exciting areas of cell biology. Fortunately several excellent in-depth reviews are presently available (4–6). The generally accepted mechanism for hormonal activation is as follows: A hormone binds to a tissue-specific receptor on the plasma membrane. The hormone-receptor complex is then functionally coupled to the adenylate cyclase. An interaction of the coupled complex with guanine nucleotides results in an activated adenylate cyclase. In many instances the activation of adenylate cyclase produces an autoregulatory process that results in a diminution of the cell's ability to respond to further stimuli. Several mechanisms are described that are probably responsible for this latter phenomenon.

Receptors and Hormone Binding

The development of radiolabeled hormones and neurotransmittors of high specific activity has led to the demonstration that most if not all circulating hormones bind to high affinity sites on the plasma membrane. Technical advances in the binding assays have enabled investigators to demonstrate that a dynamic modulation of

receptor number and affinity occurs under certain conditions (5,6). Studies in which the receptor occupancy, adenylate cyclase activity, and biologic effect of the hormone were determined have indicated that in some cell types spare receptors exist on the plasma membrane; that is, hormone occupancy of much less than 100% of the receptor sites produces a maximal biologic effect (6). "Down regulation," the apparent disappearance of receptors from the cell surface, occurs in some experimental circumstances and is discussed in a later section. In addition, several examples of an increase in receptor number have also been reported. These include positive effect of FSH on LH receptors (7) and an increase in cardiac β-adrenergic receptors during experimental thyrotoxicosis (8). Therefore hormonal effects are apparently operable at the earliest biochemical step, the binding to membrane receptors.

Receptors are probably a heterogeneous population of molecules that contain or interact with several different membrane components, e.g. phospholipids, carbohydrates, and protein (6). Purification will be necessary to understand the structure-function relation of the hormone receptor complex, but several studies indicate that different domains of hormone molecules may play a role in binding and subsequent activation of the adenylate cyclase (9). An additional area of recent interest is the mechanism by which cholera toxin stimulates adenylate cyclase in a tissue nonspecific manner. One of the toxin's subunits seems to be necessary for plasma membrane attachment to a specific ganglioside (GM_1) while another activates adenylate cyclase. While thyroid stimulating hormone shares a partial sequence homology with cholera toxin and also seems to have GM_1 as an integral part of its receptor (10), it is unlikely that all polypeptide surface receptors share a common active site.

Coupling

One of the central questions concerning hormonal control of adenylate cyclase activity is whether or not the receptor and the cyclase reside on the same or different molecules. The fact that some cells have the capability of responding to several different hormones with an increase in cAMP production has led to the framing of the "mobile receptor hypothesis" (5). Stated simply, the hypothesis holds that a complement of cyclase molecules is capable of being activated by a number of different hormone receptor complexes which diffuse laterally in the membrane until they couple with the enzyme. Several reports support at least one tenet of this hypothesis, that is, the cyclase and the receptor are separable molecular entities. Mutant lymphoma cells have been selected using one hormone that lacks a functioning cyclase response to all hormones that normally stimulate these cells (1). More recently an ingenious experiment using cell hybridization techniques has provided a reasonably definitive answer to this question. Two cell lines were prepared, one containing β-adrenergic receptors without a functioning adenylate cyclase and the other expressing adenylate cyclase activity without any detectable β-receptor. Following cell fusion the hybrid cells contained a functioning β-adrenergic–responsive adenylate cyclase (2). Subsequently two groups have solubilized, separated, and determined the molecular size of the β-receptor and the adenylate cyclase (11, 12).

Thus the two components appear to be separate. Additionally, receptor type and content may vary during the cell cycle or during development (13). This type of modulation may constitute an additional cell-specific regulatory mechanism for altering cAMP responsiveness.

Guanyl Nucleotides

The coupling of hormone-receptor complexes and subsequent activation undoubtedly is a multistep process. Insight has been gained into at least one of the determinants of the process through experiments that have described a guanine nucleotide regulatory site on the adenylate cyclase. Guanine nucleotides are capable of activating adenylate cyclase by themselves. This ability does not require that the nucleotide donate phosphate groups but rather it seems to represent allosteric regulation (14). The use of nondegradable guanine nucleotides has demonstrated a prolonged activation of adenylate cyclase by the nucleotide as well as an enhancement of hormone stimulation (14, 15). The precise molecular details of this process are under investigation but the availability of guanine nucleotide and the effect that the hormone receptor complex may have on this availability may be a key regulatory juncture in the action of both hormones and cholera toxin. In addition, in at least some adenylate cyclase systems the guanine nucleotide concentration appears to have another effect, i.e. an alteration in the binding affinity of hormone to the receptor (16).

Effect of Calcium on Adenylate Cyclase

Interrelationships between the putative intracellular messengers have been proposed. These interactions by themselves may be the basis of the complexity observed (and probably necessary) in regulating biologic systems (17). One such consideration is the effect that Ca^{2+} has on cAMP production. While Ca^{2+} seems to have an inhibitory effect on adenylate cyclase in broken cell preparations, data demonstrate that in whole cell preparations an increase in the Ca^{2+} enhances the hormonal response. A reasonable interpretation of these data by the authors holds that Ca^{2+} is involved in the transmission of the signal; that is, Ca^{2+} is important in the coupling process (18). In other experiments the interaction of Ca^{2+} with a specific Ca^{2+}-binding protein has been shown to activate adenylate cyclase (19, 20) in vitro. Further studies have demonstrated that this Ca^{2+} dependent regulator is the same as the protein that when complexed to Ca^{2+} activates phosphodiesterase (19) (see section on cyclic nucleotide phosphodiesterase). Thus, this protein in conjunction with Ca^{2+} seems capable of providing yet another level of control to cyclic nucleotide metabolism, a dual regulation of adenylate cyclase and phosphodiesterase.

Decreases in Hormonal Responsiveness

One of the teleologically attractive features of cyclic nucleotides as second messengers is that the messenger is short-lived because of rapid hydrolysis by phosphodiesterase. Therefore, the signal constitutes a rapid-on and rapid-off biochemical regulator. Further investigation has revealed another level of control, i.e. the regula-

tion of the hormone responsiveness of adenylate cyclase. At least four separate mechanisms have been described by which this type of autoregulation might occur. This mechanism would operate to prevent continual stimulation of regulatory processes.

The first example has been termed *down regulation of specific hormone receptors.* The observation is that occupancy of hormone receptors by tissue-specific agonists produces a decrease in the measurable concentration of that agonist's surface receptors. Within the subset of hormones thought to act through cAMP, this phenomenon has been described for both polypeptide hormone (21) and catecholamine receptors (22). While some differences in the down regulation of these two types of hormone receptors exist, the effect of occupancy of a high percentage of receptors is the same, a decrease in the number of hormone receptors. A somewhat different mechanism of desensitization has been described in astrocytoma cells which respond to both prostaglandins and catecholamines. In these cells the stimulation of the cell by one agonist reduces the adenylate cyclase responsiveness to both agonists. This process does not seem to require the elaboration of a diffusible inhibitory substance but rather appears to involve a less effective coupling of receptor and cyclase (23).

The last two examples of inhibition of adenylate cyclase response involve the elaboration of factors that inhibit the enzyme's activity. A feedback regulator has been characterized from adipocytes, a cell type that responds to multiple hormones. The regulator appears to decrease cyclase activity by a process involving phosphorylation of membrane proteins. This regulation is global in nature in that the regulator induced by one agonist impairs the cyclase responsiveness to other agonists as well (24). Another inhibitor has been described which arises from perfused rat liver. This low molecular weight inhibitor blocks the activation of adenylate cyclase by hormones in several different tissues. While the action of these two inhibitors is similar, the chemical characteristics differ (25).

It is not clear whether or not this type of regulation of cAMP production can affect the compartmentalization of cAMP within a single cell. However, in the section on compartmentalization research techniques evidence is presented which implies that adenylate cyclase can be activated in selective regions of certain cells. With these autoregulatory phenomena in mind it is conceivable that localized activation of adenylate cyclase on one portion of the membrane might be subject to eventual inhibition. The results cited above also emphasize the diversity of control of the adenylate cyclase response from tissue to tissue, a fact that may in part explain the tissue specificity of hormone responsiveness.

GUANYLATE CYCLASE

There has been a strong tendency to search for analogy in the metabolism of cGMP and cAMP. The intellectual allure of cAMP's well-understood adenylate cyclase and protein kinase systems was strengthened by early studies that showed hormone responsive alterations in cGMP tissue levels and cAMP-like metabolic effects of cGMP (26). However, further studies in most areas of cGMP metabolism have

shown a divergence from the cAMP analogy. Since the description of the guanylate cyclase in 1969 (27–29), the weight of experimental evidence has indicated that this enzyme's activity is not regulated by direct interaction with hormones. Reviews by Kimura & Murad (30, 31) have detailed the early progress in understanding the regulation of guanylate cyclase.

Differences Between Adenylate and Guanylate Cyclase

An initial question was whether the adenylate cyclase and the guanylate cyclase were the same molecules. This seems unlikely for several reasons. Sodium fluoride (NaF), a known in vitro activator of adenylate cyclase activity does not enhance guanylate cyclase activity (28, 32). Conversely, incubation of the particulate fraction of cells with non-ionic detergents such as Triton X-100 ® stimulates guanylate cyclase activity, but diminishes adenylate cyclase activity (32, 33). The metal ion cofactor preference exhibited in vitro by the two cyclases differs as does the tissue-to-tissue activity ratio (32, 34). Lastly the guanylate cyclases can be distinguished from adenylate cyclase by sucrose density centrifugation (35). Thus, the catalytic abilities reside in separate molecules, a fact which would not preclude one enzyme from generating the product of the other under certain conditions. An example of altered catalytic specificity of guanylate cyclase has been recently reported (36) (see below).

Perhaps the most important difference between the two enzymes, and the one most central to the present review, is their response to hormones and neurotransmitters. Adenylate cyclase is stimulated by a variety of tissue-specific hormones in vitro. In contrast, while several early reports of direct activation of guanylate cyclase exist, further work has not substantiated these findings (32, 34, 37) or has shown that the activation is due to contamination in the hormonal preparation (38). Thus, indirect hormonal regulation of the moment-to-moment activity of guanylate cyclase has been postulated. An important example of this type of mechanism was first reported by Schultz et al (39). Rat vasa deferentia were incubated with acetylcholine in a Ca^{2+}-containing medium; a rapid increase in cGMP occurred. When Ca^{2+} was omitted from the medium, no increase in cGMP was detected. It has been concluded that acetylcholine produced an influx of Ca^{2+} into the cell, which in turn through an unknown mechanism activated guanylate cyclase.

Subcellular Distribution of Guanylate Cyclase

This indirect hormonal stimulation of guanylate cyclase is best understood in light of knowledge concerning the subcellular distribution of the enzyme. The original observations that guanylate cyclase can be found either in the soluble or particulate fraction of the cell (27–29) have been extended to many tissues, and the results show that a partitioning of the enzyme activity exists that varies from tissue to tissue (31). The ratio of soluble activity (assessed in the supernatant from 105,000 X g centrifugation) to the particulate activity (measured in resuspended pellets from a 105,000 X g spin) ranges from 90% particulate in rat small intestine (27) to at least 80% soluble in rat lung and liver (32, 33). Moreover, reports have shown that when tissues are separated into the component cell types (brain-neurons versus glial elements) (40) or structural units (kidney-glomeruli versus tubules) (41), marked

differences in guanylate cyclase distribution (soluble to particulate ratio) are observed. The cumulative data indicate that the intracellular distribution is cell-specific.

In addition, particulate preparations have been subfractioned and the enzyme activity has been assessed. Particulate activity is detected in the plasma membranes and microsomal fractions of liver (42). The localization was meticulously documented by electron microscopy and assay of marker enzymes. Studies conducted in this laboratory have demonstrated that guanylate cyclase is present in purified rat liver nuclei (43). A similar finding has been reported in the uterus (44). The localization of guanylate cyclase in specific membranes and organelles may have regulatory significance; for example, extracellular effectors may act indirectly to modulate locally the activity of a compartmentalized guanylate cyclase. A similar hypothesis of activation of compartmentalized adenylate cyclase in the lymphocyte has recently been framed by Parker (45).

The distinction between the soluble and particulate guanylate cyclase rests on several differences in their biochemical properties. Detailed kinetic analysis comparing the soluble enzyme with the detergent-dispersed-particulate enzyme showed that the soluble enzyme exhibited classic Michalis-Menton kinetics with respect to MnGTP, while the particulate enzyme exhibited positive cooperativity (32, 33). The possibility of kinetic heterogeneity of the particulate enzyme has been raised (46) but in general the kinetics are distinct from those of soluble enzyme. Additionally the two forms were shown to be different molecular sizes by gel filtration (32, 33) and by sucrose density gradient centrifugation (35). These reports do not rule out the existence of a core catalytic unit with additional subunits that confer the distinctive size and kinetic properties. In fact, when sea urchin particulate guanylate cyclase was purified (47), the kinetic properties were altered from the cooperative kinetics observed in the crude preparation to soluble-like kinetics in the purified preparation. It is unknown at this time whether this shift in kinetic properties is an artifact of preparation.

Regulation of Guanylate Cyclase Activity

Comparison of experiments done in tissue slices with in vitro assays of guanylate cyclase activity indicates that several determinants of enzyme activity exist. Included are Ca^{2+}-dependent mechanisms and Ca^{2+} independent mechanisms, e.g. modulation of activity by adenine nucleotides, oxidation-reduction reactions, and fatty acids. The Ca^{2+}-dependent regulation is implied by the work of Schultz et al (39) in which the stimulation of cGMP levels by acetylcholine in the isolated vas deferens was dependent upon Ca^{2+} fluxes. Ca^{2+}-independent mechanisms have been documented in the human umbilical artery where serotonin increases cGMP in Ca^{2+}-free media (48). These experiments give a basis for the extrapolation of conclusions based on kinetic analysis of enzyme activity to a theory of regulation in a complex cellular milieu. When soluble guanylate cyclase is assayed in the presence of suboptimal but physiologic Mn^{2+} concentrations, mM concentrations of Ca^{2+} can increase the activity of the enzyme (32, 33); Mg^{2+} can also enhance activity under these conditions (42). The particulate enzyme exhibits an even more complex behav-

ior with respect to Ca^{2+}. Crude particulate activity is inhibited by mM concentrations of Ca^{2+} (32, 33) while the purified microsomal guanylate cyclase is unaffected (42). However, the detergent-treated fibroblast particulate guanylate cyclase is stimulated by μm Ca^{2+} in the presence of low Mn^{2+} and 3 mM Mg (49). S. Levine and G. Meisner (unpublished results) working in this laboratory have shown that purified sarcollema from rabbit muscle contains a guanylate cyclase which is stimulated by μM Ca^{2+} in the presence of low Mn^{2+} and 3 m Mg^{2+}. This effect is reversed when the enzyme is solubilized with detergent. These data imply that Ca^{2+} can in some fashion modulate the activity of both forms of guanylate cyclase and that hormonal alteration of the localized Ca^{2+} concentration might directly alter cGMP production.

Regulation by guanine nucleotides of the adenylate cyclase seems to have a guanylate cyclase counterpart. Physiologically relevant concentrations of ATP inhibit both the soluble and particulate guanylate cyclase (32, 33). Kinetic analysis indicates that a metal-adenine nucleotide site exists on the guanylate cyclase which may participate in bidirectional regulation (50). In another system the activity of the nuclear guanylate cyclase is modestly stimulated by cAMP in vitro but it is unclear whether this effect is a direct one on the enzyme or is mediated by cAMP-dependent protein kinase (43). Thus, hormonal modulation of nucleotide pools and cAMP may also affect the activity of the guanylate cyclase.

Murad and co-workers (51) have pursued a serendipitous observation that sodium azide (NaN_3) activates guanylate cyclase and have uncovered another possible regulatory mode. Preincubation of guanylate cyclase preparations from some, but not all, tissues with NaN_3 results in up to a 30-fold increase in enzyme activity. The activation by NaN_3 produced additional changes in the properties of the enzyme including an alteration in the metal cofactor requirements (52) and substrate specificity (36). Murad et al were able to partially purify from liver a protein factor necessary for activation. Transfer of this factor to a soluble guanylate cyclase preparation from brain allowed NaN_3 activation, whereas prior to addition of the factor none was detectable (53). Further data have been presented which tentatively identifies the NaN_3 activation factor as the enzyme catalase (54, 55). The sequence of events seems to include the conversion of NaN_3 by catalase to a nitric oxide–containing compound. In fact, nitric oxide (NO) by itself has been demonstrated to produce activation (56). The importance of this mechanism is emphasized by recent work which shows that both the vasodilator nitroprusside (56) and certain carcinogens such as nitrosoguanidine (57) activate guanylate cyclase.

The importance of oxidation and reduction reactions in the activation of guanylate cyclase by NaN_3 probably has wider applicability. Autoactivation of soluble guanylate cyclase from some tissues occurs in the presence of O_2 and can be mimicked by H_2O_2 under certain conditions (58). A direct effect of fatty acids on the activity of soluble guanylate cyclase has also been observed (59). This direct activation by specific fatty acids may also be dependent on the oxidation and reduction status of the tissue. In addition, the stimulation of membrane-bound activity by fatty acids has been observed (60, 61). It is unclear whether this represents a direct effect on enzyme activity or a further solubilization of latent enzyme activity. The final

common pathway by which these diverse mechanisms alter enzyme activity is unknown but offers a challenging problem for future study.

Regulation of Guanylate Cyclase Concentration

The mechanisms discussed above demonstrate that the acute regulation of guanylate cyclase activity is probably a localized phenomenon which depends upon a very complex set of physical and chemical interactions. Hormones and neurotransmittors undoubtedly affect the activity of the enzyme but do not do so directly. It is logical to assume that in addition to the acute regulation of existing guanylate cyclase activity, mechanisms exist by which the total concentration of the different forms of guanylate cyclase are modulated. Several examples have been studied that involve the processes of growth, development, or involution of a tissue. During rat liver regeneration there is a two- to threefold increase in the particulate guanylate cyclase activity (62–64). In hepatic development the particulate activity of fetal liver is higher than that of the adult; thus the regenerating liver reproduces the ratio seen in the fetal liver (62). Early during testicular development all forms of guanylate cyclase activity are elevated when compared to adult levels, but the soluble activity remains elevated later into development (65). Testicular atrophy produced by unilateral cryptorchidism produces a sustained elevation of the soluble activity (65). These examples demonstrate that the two major forms of guanylate cyclase may be regulated independently, a fact that would allow flexibility in the compartmentalization of the enzyme.

The relationship of the partitioning of the guanylate cyclase to the resting tissue level of cGMP is an additional area of interest. The examples studied to date indicate that this relationship may be tissue-specific. In the rat kidney, cGMP levels in different anatomic regions of the kidney are best correlated with the particulate activity of the region (66). In the testis, increased levels of cGMP are clearly associated with an increase in the soluble activity (65). During liver regeneration the increase in particulate guanylate cyclase activity is not correlated with any overall change in tissue cGMP concentrations (64, 67) but rather is associated with an enhanced localization of cGMP on cellular membranes as adjudged by immunofluorescent analysis of regenerating liver (64).

In conclusion, the guanylate cyclase exists in several different forms which are localized to certain intracellular sites. Modification of the subcellular distribution and total enzyme concentration have been described. The acute regulation of enzyme activity probably depends upon the local interaction of ions, nucleotides, oxides, and fatty acids with the guanylate cyclase. Thus, the production of cGMP by guanylate cyclase is most likely a compartmentalized biochemical phenomenon that indirectly responds to hormones and neurotransmittors.

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

The regulation of cyclic nucleotide metabolism by phosphodiesterases has been extensively studied during the last few years. Of particular interest are selected papers that give an overview of the role of phosphodiesterases in modulating cyclic

nucleotide levels at key intracellular sites. The role of calcium and its interaction with the calcium dependent regulator protein is emphasized below.

Multiple Forms of Phosphodiesterase

Numerous workers have shown that cyclic nucleotide phosphodiesterase activity in tissues and cells is comprised of multiple enzyme forms. These phosphodiesterases differ in their site of distribution within tissue (i.e. membrane-bound or soluble), in their specificity for hydrolysis, and in the avidity of the enzymes for their substrate(s). The classification by Appleman, Thompson & Russell is widely used (68). These authors showed that rat liver extracts, chromatographed on DEAE-cellulose columns, exhibited three phosphodiesterase activities. Fraction I hydrolyzed cGMP, was unable to hydrolyze cAMP, and was not inhibited by cAMP. This fraction is found in the soluble extracts of the cell and is activated by the calcium dependent regulator (69) (see below). Fraction II hydrolyzed both cAMP and cGMP with relatively low substrate affinities. This fraction was the predominant one in rat liver extracts, and at pH 7.4 cAMP hydrolysis was activated by low concentrations of cGMP (68, 70). Fraction III was a high affinity enzyme which hydrolyzed cAMP, but not cGMP, was associated with membrane fractions, and was inhibited by cGMP in a hyperbolic fashion (68, 70).

Recently, Pichard & Cheung (71) have reported the separation of multiple forms of phosphodiesterase(s) from rat liver and human blood platelet. Using sucrose density gradient centrifugation, they found that the multiple forms of cAMP dependent phosphodiesterase that were isolated were intraconvertible. The equilibrium between these forms was altered in vitro by enzyme concentration and by dibutyryl cAMP. In addition, phosphodiesterase activity and apparent K_m were affected by enzyme concentrations (71). The authors showed that upon recentrifugation on sucrose gradients each form of cAMP phosphodiesterase could be generated from the others. These authors imply that the multiple forms appeared to represent different aggregated states of the enzyme. They found that the kinetics of hydrolysis for both cyclic nucleotides could be changed by the enzyme concentration. Cyclic GMP hydrolysis was affected by association and dissociation in a manner that was opposite to that of cAMP, suggesting that cGMP phosphodiesterase is probably separate from cAMP phosphodiesterase. They also hypothesized that since dibutyryl cAMP induced a shift towards the dissociated form, which is more active for cAMP, a self-compensating mechanism to hydrolyze excessive cAMP following stimulation might exist. Additional work is needed to definitively determine the number, specificity, and location of the phosphodiesterases.

The Ca^{2+} Dependent Regulator Protein and Cyclic Nucleotide Metabolism

Another important factor that can affect phosphodiesterase activity is the activator protein originally described by Cheung (69). This protein specifically binds Ca^{2+} and after binding undergoes a conformational change. When associated with Ca^{2+} an active phosphodiesterase-activator complex is formed (72, 73). The calcium dependent regulator (CDR) selectively increases the activity of cytosolic but not mem-

brane-bound phosphodiesterase, and does not appear to affect the equilibrium among the intraconvertible forms of the phosphodiesterase described above (71). The CDR is also capable of increasing the activity of adenylate cyclase (19). CDR from bovine brain and bovine heart have a remarkable sequence homology to the calcium binding subunit of muscle troponin (74, 75) a fact that suggests a common phylogenetic origin. The muscle protein, troponin C, has a regulatory role in the interaction between actin and myosin during contraction. Other investigators have described a similar Ca^{2+} binding protein in the adrenal medulla (76). Since actomyosin complexes have been shown to exist in adrenal medulla (77) an intriguing possibility exists, a common mechanism of Ca^{2+} action. Ca^{2+} through its binding to a ubiquitous class of binding proteins, could modulate not only the synthesis and hydrolysis of cyclic nucleotides, but could also regulate contractile events in cells including stimulus-secretion coupling.

An example of potential compartmentalization control of cAMP levels by the CDR has been demonstrated in the rat adrenal medulla. When postsynaptic nicotinic receptors were activated transsynaptically, cAMP content increased and the CDR of cyclic nucleotide phosphodiesterase increased in the soluble fraction (78). Initially CDR might serve to enhance the cAMP response to synaptic transmission. The subsequent movement of the activator from the membrane to the soluble fraction might be explained by a cAMP-mediated release from the membrane. In fact, the authors found that cAMP-dependent protein kinase, in the presence of ATP and cAMP, stimulated the release of the protein activator from a particulate fraction of adrenal medulla (78). The soluble CDR might then increase hydrolysis of both cyclic nucleotides. CDR stimulates both cAMP and cGMP phosphodiesterase, but the K_m of activation is significantly lower for cGMP than cAMP (79). Consequently, in the presence of hormones that increase cAMP levels, the CDR might first accelerate cAMP synthesis and then cause a rapid hydrolysis of cGMP at a specific intracellular site. On the attainment of significantly elevated cAMP levels, the calcium activator complex might then accelerate the hydrolysis of cAMP, restoring the level of the nucleotide at the site back towards normal. The experiments that imply translocation of CDR suggest a mechanism that might control the level of the two cyclic nucleotides at particular intracellular sites.

Hormones and Phosphodiesterase

Certain hormones can activate phosphodiesterase in vivo but not in vitro. Insulin activated phosphodiesterase in intact fat cells (80, 81) by increasing the V_{max} value of the low K_m phosphodiesterase which was found in a particulate fraction (81). In a later study (82), the authors found that insulin in vivo doubled the phosphodiesterase activity of subsequently isolated fat cell membrane fractions. This stimulation occurred in seconds and could be inhibited by ethyleneglycol-bis(β-smiroethyl ether)-N,N' tetraacetic acid (EGTA) or SH compounds (82). The activation of phosphodiesterase by insulin does not appear to involve CDR since the inhibitory effects of EGTA were not overcome by the addition of Ca^{2+}. Norepinephrine also activated fat cell phosphodiesterase in a manner similar to that of insulin (82, 83). These studies indicate that hormones can activate phosphodiesterases by some

unknown mechanism. Because insulin does not stimulate phosphodiesterase activity in broken cell preparations (80, 81), the possibility of an intracellular messenger for this hormone action seems likely.

CYCLIC AMP-DEPENDENT PROTEIN KINASE

Recent progress in the area of cAMP-dependent protein kinases has centered on the classification of the forms of protein kinase, the molecular mechanism of regulation, the isolation of natural substrates, and the possible translocation of subunits after activation by hormones. There is a consensus that at least two types of soluble cAMP-dependent protein kinases exist in mammalian tissues. These are separable by column chromatography (84, 85). Each isozyme of soluble protein kinase forms a tetramer composed of two regulatory and two catalytic subunits. The two isozymes differ in the characteristics of their receptor subunits (R_1, R_2), while the catalytic moiety (C) appears to be identical in structure and function (84, 86–88). Activation of catalytic activity is seen in each when cAMP binds to the regulatory subunit and dissociates R and C. The catalytic unit freed from inhibition by R then phosphorylates specific serine residues in histone and other protein substrates (89). The phosphorylation can be inhibited in the presence of a protein kinase inhibitor protein (89).

Properties of the cAMP-Dependent Protein Kinases

When the two isozymes are compared, Type I has a lower molecular weight, binds ATP with higher affinity, and does not appear to be autophosphorylated (see below) (84, 85, 87). In addition, R_1 and C are rapidly dissociated into subunits by histone or high salt (0.5 M) and reassociate slowly in dilute buffer (85, 87). The isozymes appear to differ in their distribution in mammalian tissues. For example, Type II isozymes predominate in beef or guinea pig heart (87, 90), while rat and mouse cardiac tissue contain 75 to 80% of Type I (84).

Both isozymes are activated by concentrations of the nucleotide which are in the physiological range (91). Modification of the cAMP requirement for dissociation occurs with binding of Mg^{2+} ATP to the regulatory subunit of Type I isozyme; this increases the concentration of cAMP required for activation (92). The cAMP requirement is also altered by the concentrations of protein kinase in tissue (92), and the autophosphorylation of the receptor subunit of the Type II isozyme (93). Phosphorylation of the R_2 by the catalytic unit increases the sensitivity of the Type II protein kinase to activation by cAMP (94) and slows the reassociation process which leads to inactivation (94). A contrasting action of cAMP is that it stimulates the activity of a phosphatase which can dephosphorylate the R_2 subunit (95). These findings imply a fascinating mechanism of control in which cAMP regulates both the disassociation and reassociation of the Type II protein kinase.

A possible third form of protein kinase has been identified in membrane fractions of bovine brain, heart, and muscle solubilized with Triton X-100 (96). These membrane-derived enzymes chromatographed separately from the two cytosol enzymes from the same tissue. However, protein kinase activity from the solubilized liver

membranes cochromatographed with one of the liver cytosol protein kinases. In brain, the membrane-derived protein kinase preferred protamine to histone as substrate, and seemed to have a catalytic unit which sedimented at a different coefficient from the cytosol catalytic unit (96). Other investigators (87) have questioned whether this membrane-derived protein kinase enzyme might not be a form of Type II isozyme, because the Type II isozyme also appears to bind tightly to membrane components. It will be important to resolve the question whether the soluble protein kinases differ in their properties from the membrane kinases. The reported homogeneity of the catalytic subunit of protein kinase from several species and tissues would tend to argue against a different form of the catalytic subunit in membranes, but a membrane catalytic unit of different specificity would allow a greater diversity of control.

Accumulated evidence has shown that the cAMP-dependent protein kinase phosphorylates serine residues in many intracellular substrates (97). Denaturation of certain proteins leads to phosphorylation of the protein by protein kinase that did not occur in the native state (98, 99). Therefore, protein tertiary structure seems to be important in substrate specificity. Recent studies have shown that the presence of two adjacent basic amino acids on the N-terminal side of the susceptible serine residue may be important for specific substrate recognition in vivo (99, 100). While these observations suggest that a common structural requirement is necessary for phosphorylation, it will be important in future work to identify the preferred substrates for protein kinase in vivo, as well as to determine the factors that can modify the susceptibility of these proteins to phosphorylation. For example, phosphorylase kinase can be phosphorylated to much higher levels when the Mg^{2+} concentration in the incubation medium is increased from 2 mM to 10 mM (101). The authors postulated that the higher Mg^{2+} concentration may be altering the conformation of phosphorylase kinase thereby rendering more phosphorylation sites accessible to protein kinase (101). These observations suggest an intriguing mechanism for regulation in which changes in the intracellular levels of ions could modulate the extent of phosphorylation and perhaps the functional properties of the molecule. For example, when glycogen synthetase was phosphorylated to different extents, it exhibited different regulatory properties (102). These observations imply that regulation by protein phosphorylation may be an interrelated process that depends not only on changes in the level of cAMP at intracellular sites, but also on levels of cations and other factors.

Measurement of the concentration of the receptor and catalytic subunit in a number of tissues has shown that the two subunits exist in a 1:1 molar ratio (103). Furthermore, it was noted that the molar concentration of the receptor subunit of protein kinase closely approximated that of cAMP under basal conditions (103). This close correlation between the concentration of cAMP and protein kinase subunits would allow activation of the enzyme by small changes in the nucleotide at specific intracellular sites. In these studies, almost all the protein kinase subunits were found in the soluble fractions, except for brain where 40% of activity was particulate (103). In another study, washed particulate fractions from rat heart contained approximately 20% of the total activity, while in rabbit heart this fraction

contained nearly half the total enzyme (87). Clearly, additional work is needed to definitively determine the intracellular location of the isozymes.

Protein Kinase Inhibitors

A protein that inhibits the activity of cAMP-dependent protein kinase was first described by Walsh et al (89). This small protein is found in many tissues, is thermostable, and binds to the catalytic subunit of protein kinase but not to the holoenzyme (89). The physiologic significance of the protein is unknown but by binding to the catalytic subunit the protein inhibits cAMP-dependent phosphorylation directly, and thus may require the generation of larger amounts of cAMP for persistence of a cAMP-mediated biological response. The presence of this protein in one area of the cell could also block the action of cAMP at specific sites while allowing the expression of cAMP action in the rest of the cell. Furthermore, it is possible that cAMP bound to receptor subunits of protein kinase has biological activity in itself (see below). The inhibitor protein could thus function to block the activity of the catalytic unit and leave the receptor cAMP complex free to perform a biologic function.

Recently, a second endogenous inhibitor protein has been identified (104). It is also thermostable, is smaller than the protein described above, and inhibits protein phosphorylation catalyzed by several types of protein kinases (cAMP- and cGMP-dependent or cyclic nucleotide-independent protein kinases). This protein has been purified 1500-fold from rat brain, and apparently acts by competitively antagonizing the interaction between protein kinase and the phosphate acceptor protein. This new inhibitor protein (Type II inhibitor) was found in several tissues and was present in varying amounts in these tissues (104). In addition, the ratio of amount of Type II inhibitor to Type I [the protein originally described by Walsh et al (89)] varied from tissue to tissue (104).

Translocation of Protein Kinase Subunits

Recently attention has been paid to the possible translocation of the catalytic and perhaps the receptor subunits from one intracellular site to another following dissociation of the holoenzyme. Translocation of the catalytic subunit from cytoplasm to nucleus has been suggested in the liver (105, 106), adrenal medulla (107), and ovary (108). General evidence for translocation to nucleus in these systems has included a time-dependent decrease in the activity of cytosolic protein kinase with increased activity in particulate fractions. For example, in adrenal medulla, prolonged stimulation of nicotinic receptors was associated with increased tissue levels of cAMP and increased cytosolic protein kinase activity. Over time, a decrease in cytosolic activity and an increase in activity in particulate fractions, including purified nuclei, were observed (107). The translocation of the catalytic subunit to nucleus was correlated with the induction of tyrosine-3-monooxygenase (107). In another system nuclei isolated from rat liver perfused with glucagon contained three times as much protein kinase activity as did nuclei from controls (105). In the presence of either the heat-stable inhibitor or the protein kinase regulatory subunit,

the elevated cAMP-independent enzyme activity from stimulated nuclei was inhibited to an activity equivalent to that found in control (105). These findings are reasonable in light of the evidence of in vivo phosphorylation of nuclear histone H1 in response to glucagon (109).

Translocation of receptor subunits into the nucleus has been suggested for porcine ovary (108). In a recent report a cAMP-dependent protein kinase identical with that found in cytoplasm was isolated in porcine ovary nuclei (110). The enzyme bound cAMP, suggesting an intranuclear localization of receptor protein. These data suggest that translocation of both subunits can occur. Using a different methodological approach, that of photoaffinity labeling of N^6-butyryl cAMP to a cytosolic receptor protein, Kallos (111) has demonstrated that the cAMP-receptor complex is translocated into the nucleus. The nuclear binding of the receptor complex seemed to be noncovalent, since the N^6-butyryl cAMP complex is extractable from the nucleus with 0.4 M KCL. When rat liver nuclei were incubated at 25°C with ^3H N^6-butyryl cAMP in the presence of cytosol, there was a time-dependent binding of the radioactivity to the nucleus; in the absence of cytosol, the label bound poorly to the nuclei. While far from definitive, the above results also suggest that the cAMP receptor unit may play additional physiological roles. This mechanism of translocation would resemble the translocation of steroid hormone cytosolic receptor complexes. A similar nuclear role for the receptor unit should be sought.

In preliminary studies in our laboratory using antibodies to receptor and catalytic subunits of protein kinase isozymes I and II, a technique has been developed to perform immunocytochemical localization of these subunits. Increased fluorescence of both the receptor and catalytic subunits in rat liver nuclei has been detected during rat liver regeneration (112). Protein kinase holoenzyme was not visualized in nuclei. This immunocytochemical approach should be helpful in elucidating the sites of localization of these protein kinase subunits following peptide hormone administration.

Site-Specific Function of cAMP-Dependent Protein Kinase

An ingenious experiment has been designed that delineates a possible role for the catalytic unit in nuclear regulated processes. Maller & Krebs (113) have shown in ripe *Xenopus* oocytes that the microinjection of high purified preparations of regulatory and catalytic subunits of cAMP-dependent protein kinase from muscle modified the progesterone-induced meiotic cell division. Microinjection of the catalytic subunits at a specific time point after the second meiotic metaphase inhibited the progesterone-stimulated cell division sequence. Conversely, microinjection of the regulatory subunit in the absence of progesterone induced division (113). The authors postulated that the activity of the catalytic subunit prevented progress of the oocyte through meiotic cell division (113). They presented a model in which a high steady state level of cAMP dependent protein phosphorylation by the catalytic subunit maintained prophase block of the oocyte (113). Frog oocyte provides an excellent model for examining in detail the role of cAMP and the protein kinases in nucleus-directed events.

Cyclic GMP–Dependent Protein Kinase

Cyclic GMP–dependent protein kinases have been reviewed in this volume (114). It is not clear at this time whether all of the biologic activities of cGMP are mediated through the activation of cGMP-dependent protein kinase. The enzyme appears to be different from cAMP-dependent protein kinase because the receptor and catalytic subunits are not dissociated upon activation by cGMP (115, 116), although other investigators have shown disassociation by cGMP in certain tissues (117). Immunocytochemical studies using antibodies to cGMP in a variety of tissues have shown localization of the nucleotide on plasma membranes and nuclear elements, and in cytoplasm (118). Immunochemical studies utilizing antibodies to the cGMP-dependent protein kinase would be most helpful in providing clues as to the location of the natural substrates of the enzyme and thus might help to elucidate the roles of cGMP in cell function.

COMPARTMENTALIZATION AND ALTERNATE SECOND MESSENGERS

During the last several years, some doubt has been raised as to whether cAMP is in fact the second messenger for hormones in specific tissues. The most carefully examined systems include the action of adrenocorticotropic hormone (ACTH) in promoting steroidogenesis in adrenal cortex (119); the action of luteinizing hormone (LH) in stimulating steroidogenesis in ovary and testis (120); the stimulation of glycogenolysis by catecholamines in liver (121–123); and the stimulation of amylase, electrolyte, and water secretion in acinar cells of parotid gland by catecholamines and cholinergic agents (124).

Does cAMP Mediate Steroidogenesis?

Sutherland and colleagues established four criteria for evaluating whether or not cAMP functioned as a second messenger in a particular hormone action (125). Experimental data on the regulation of steroidogenesis by ACTH and LH have satisfied these criteria in the respective target tissue. However, in the early 1970s, it became evident that both in adrenal cortex (126–128) and gonadal tissues (129, 130), steroidogenesis could be activated by low concentrations of hormone without any detectable change in the tissue concentration of cAMP. These studies thus raised doubts whether cAMP was the second messenger for steroidogenesis.

To examine this question, the effect of LH and human chorionic gonadotropin (hCG) on testosterone production in homogeneous populations of collagenase-prepared Leydig cells has been studied. A dose-response curve to hCG showed that enhanced steroidogenesis occurred without any changes in cAMP levels (129–131). Cyclic AMP levels increased with higher dose of hormone, but differed by at least an order of magnitude in sensitivity when compared to testosterone production. Furthermore, when protein kinase activity was assessed in this system, utilizing histone H2b as a substrate, the dose-response curves comparing testosterone synthesis with protein kinase activation in the presence of low doses of hCG were again disparate (132). However, very recently another group has successfully correlated

protein kinase activity ratio with steroidogenesis in corpus leuteal slices (133). Assessment of cGMP during hCG stimulation did not show any correlation between cGMP levels and testosterone production (134). Thus, neither cyclic nucleotide increased in concentration with doses of gonadotropin which stimulated steroidogenesis.

This dissociation between cAMP levels and testosterone production can be interpreted in one of two ways. First, another second messenger is rate limiting for hormonal stimulation of steroidogenesis, or second, the measurement of total cellular cyclic nucleotide is not sufficiently sensitive to detect the alterations in cyclic nucleotide metabolism that take place. That the latter conclusion seems most likely is supported by the recent studies of Dufau et al (135). The problem has been approached by measuring the free and occupied sites of the cAMP binding proteins in the Leydig cells (presumably the receptor component of protein kinase). The authors observed a dose-dependent increase in cAMP binding during incubation of purified Leydig cells with low concentrations of hCG. Most important, the testosterone dose-response curve was well correlated with the increase in cAMP receptor occupancy (135). This study provides strong evidence for the role of cAMP and protein kinase during hormonally induced steroidogenesis and offers a sensitive method for examining the role of cAMP in the action of peptide hormones.

A similar approach for detecting occupancy of cAMP receptor subunits has been developed by Terasaki & Brooker (136). The concentration of free and bound cAMP was measured in isolated rat atrium utilizing a rapid high dilution technique that involved tissue homogenization, subcellular fractionation, and separation of bound from free cAMP by millipore filtration. Spontaneously beating rat atria contained 5.96 ± 0.2 pm of cAMP/mg of protein of which 41 and 14% were bound to soluble and particulate proteins, respectively. The remaining cAMP was free. Pretreatment of the atria with 1 μM isoproterenol or with a phosphodiesterase inhibitor resulted in increased binding of both soluble and particulate proteins. When both agents were used together, free cAMP increased 27-fold, while bound increased 4-fold, indicating saturation of the soluble sites. The authors calculated that fewer than one third of these sites are occupied in the unstimulated cell, and that half-maximal binding in vivo occurs at an intracellular concentration of about 1 μM cAMP (17). The results indicate that after the initial 3- to 4-fold increase in cardiac cAMP little additional binding of cAMP to receptors would be expected to occur (136). These findings substantiate the hypothesis that activation of protein kinase occurs with only minimal changes in cAMP content.

Does cAMP Mediate Glycogenolysis?

The role of cAMP in the action of glycogenolysis by catecholamines has also been reexamined during the last few years in both perfused rat liver (121, 123) and isolated hepatocytes (121, 122). In both systems it was clearly shown that the α-adrenergic agonist phenylephrine stimulated phosphorylase activation without appreciable changes in the concentration of cAMP (121–123). Furthermore, phosphorylase activation by epinephrine is probably accomplished through activation of the α-receptor in these systems because the β-adrenergic antagonist, propranolol, pre-

vented increased cAMP levels, but did not inhibit activation of the enzyme system (137). In isolated hepatocytes, phenylephrine stimulated the glycogenolytic system without activation of cAMP-dependent protein kinase, whereas an equivalent glycogenolytic concentration of glucagon elevated cAMP and increased protein kinase activity (137). These data indicated that a separate mechanism independent of cAMP was involved in α-adrenergic action in hepatocytes. In support of this concept is the observation that the activation of phosphorylase and glucose release by phenylephrine was impaired in isolated rat hepatocytes in which Ca^{2+} had been depleted by EGTA. The activation was restored by Ca^{2+} addition (138). The effects of a glycogenolytically equivalent concentration of glucagon on these processes were not altered by EGTA. The role of Ca^{2+} in these α-adrenergic processes was also supported by the fact that the divalent cation ionophore A23187 increased phosphorylase A activity and glucose output. The investigators proposed that α-adrenergic agents activate phosphorylase by increasing the cytosolic concentration of Ca^{2+} (138). While the proposed mechanism is conjectural, recent findings have shown that liver phosphorylase kinase, like the muscle enzyme (139), can be stimulated by Ca^{2+} (140, 141).

This study suggested that glycogenolysis can be achieved by alternate mechanisms: (*a*) a cAMP-dependent activation of phosphorylase by glucagon, (*b*) an activation of phosphorylase by changes in cytosolic Ca^{2+}. It will be difficult to absolutely prove the second mechanism because of technical difficulty in localizing intracellular changes in Ca^{2+} concentration. Schramm & Selinger (124) have reviewed the concept of alternative second messengers and suggest that such a system increases diversity of control. They have presented data in both parotid gland and pancreas (124).

TECHNIQUES FOR COMPARTMENTALIZATION RESEARCH

The delineation of the second messenger concept has been one of the major advances in cellular biology during the past two decades. Cells utilize these second messengers in a variety of ways to allow both flexibility and specificity of response to an external stimulus. In a specific tissue, second messengers promote not only immediate responses such as enzyme activation and secretion, but appear to play some role in more complex phenomena such as growth and differentiation. The specificity of response must logically depend not only on the signal received at the cell surface, but also on the precise temporal and spatial effect of the intracellular messenger. It may be asked, "How can such specificity be achieved when the number of recognized second messengers appear to be so limited?"

We have attempted to emphasize in this review that the component parts of the second messenger systems are compartmentalized within the cell and that this promotes the localization of cyclic nucleotides as well as flexible interrelationships with other factors involved in hormonal responses. Hormone-dependent adenylate cyclase is confined to membranes, while guanylate cyclase is both soluble and particulate. There are soluble and particulate cyclic nucleotide phosphodiesterases.

Additionally, newer information suggests that both cytosol and membrane cAMP-dependent protein kinases exist. The translocation of the subunits of cAMP-dependent protein kinase and the potential control by protein kinase inhibitor proteins would allow further intracellular specificity. Lastly, there is abundant evidence to show that divalent cations are important in the regulation of cyclic nucleotide levels and action. The ability to study the "fine tuning" by cations in vivo is limited, but intracellular compartmentalization of these charged ions is more than speculation (142). In summary, the response of cells to external stimuli is much more complex than a whole cell on or off signal dictated by the tissue concentration of a second messenger. A signal might evoke a small change in cyclic nucleotide level with a consequent response to a confined area of the cell, e.g. in the nucleus or at a particular contractile site near the cell membrane. A different or more persistent signal might produce a more global response. We would like to suggest some methodologic approaches that might provide information concerning the action of cyclic nucleotides and protein kinases. These approaches are both biochemical and cytochemical and are best utilized in combination.

Cyclic Nucleotide and Protein Kinase Immunocytochemistry

An immunocytochemical method to detect the intracellular location of both cyclic nucleotides has been developed and was recently reviewed (143). The pattern of cAMP and cGMP immunofluorescence varies from cell type to cell type; therefore, the technique is useful for detecting changes in cells of a heterogenous tissue as well as differences in intracellular localization. The localization and intensity of cyclic nucleotide immunofluorescence has been noted to change after hormonal stimulation (143). In addition perturbation of lymphocyte cell membranes by latex particles or by binding of antisera to surface immunoglobulin results in a marked localization of cAMP near the cell surface (143, 144). During liver regeneration the immunocytochemical procedure has detected changes in cGMP distribution at times following partial hepatectomy in the absence of any change in the tissue cGMP level (64, 145). Therefore we believe that in some circumstances immunocytochemistry is a more selective and powerful tool than tissue levels, albeit less quantitative. This technique should continue to provide clues to the roie of cAMP and cGMP in hormone action.

Antisera to the receptor and catalytic subunits of the cAMP-dependent protein kinases have been recently utilized in an attempt to localize these subunits by immunocytochemistry (112). The immunofluorescence pattern of the receptor subunits of isozymes I and II as well as the catalytic subunit are altered in rat liver following glucagon administration (112). The detection of increased fluorescence in the nucleus during rat liver regeneration suggested a translocation of these subunits into the nucleus from cytoplasm. This tends to confirm biochemical data cited above. Further experiments may help resolve the questions concerning the intracellular sites of action of the cAMP-dependent protein kinases, as well as help investigate any alternate role for the regulatory units. Purification of the other components of the cyclic nucleotide metabolic system will allow application of the technique to

other questions. Adaptation of the technique to the electron microscopic level is under way and should provide more precise localization of the site of action of the cyclic nucleotides.

Biochemical Measurements of Cyclic Nucleotide Compartmentalization

The newly developed biochemical techniques to assess the concentration of bound and free cAMP have been discussed in the previous section (135, 136). They show great promise in enhancing the ability to detect a small rise in the active fraction of cAMP. Similar techniques to assess cGMP binding ratios would also be useful in light of the example of liver regeneration in which cGMP localization is altered while levels stay the same. The subcellular distribution of cAMP-dependent protein kinase has also been approached from a biochemical point of view. Corbin et al (146) have shown that under certain conditions 50% of the Type II isozyme in rabbit heart is bound to membrane. With epinephrine, which raises cAMP levels, the catalytic unit is dissociated from the regulatory subunit and is released from the membrane. This and other approaches to the study of translocation of protein kinase subunits will yield valuable information concerning the site of action.

Subcellular Fractionation and Microdissection Techniques

Subcellular fractionation and/or microdissection techniques have been helpful in pinpointing the cellular location of the enzymes involved in cyclic nucleotide synthesis and hydrolysis. Elegant microdissection techniques have been applied recently to the identification of hormone-specific adenylate cyclase activity in very minute areas of rat nephron (147). Such studies have helped to identify the sites of action of both parathyroid hormone (PTH) and vasopressin in the nephron (147).

Isolation of cellular organelles such as the nucleus has significant potential. For example, rat liver nuclei possess guanylate cyclase activity (43). It would be particularly valuable to use nuclei for electron microscopic immunocytochemical localization of cyclic nucleotides and their kinases. This approach would complement studies in which phosphorylation patterns in nuclei are correlated with transcriptional activity after addition of hormones or cyclic nucleotides. The same type of approach to subcellular fractionation might yield information concerning the role of cyclic nucleotides in other organelles such as mitochondria, lysosomes, synaptic vesicles, and glycogen particles.

Determination of Phosphorylated Proteins After Hormone Administration

Because the cyclic nucleotides are believed to act through phosphorylating specific substrates (although we believe that we should keep an open mind about other potential modes of actions of the nucleotides), studies in vivo and in vitro that identify phosphorylated proteins after a defined stimulus have provided clues to the nature of cyclic nucleotide action. Systems in which the phosphorylation of specific proteins has been correlated with cAMP effects include vasopressin stimulation of sodium and water transport in toad bladder (148), catecholamine-stimulated ion flux in turkey erythrocyte (15) and in cardiac sarcoplasmic reticulum (149), and

lectin-stimulated transport function in human lymphocytes (150). In none of these systems has it been proven that phosphorylation of specific protein is the cause of a physiologic action postulated to be mediated by cAMP, but the circumstantial evidence correlating ion transport with phosphorylation is quite strong (151). In most of these systems the number of proteins phosphorylated has been surprisingly few. This discrete phosphorylation provides selectivity in control mechanism but does raise the question of how diversity of control is obtained when such a small number of proteins are phosphorylated (151).

The lymphocyte system has been reviewed recently by Parker (45). It is most fascinating because the results dramatize the idea of compartmentalization of cyclic nucleotides in lymphocyte function. In human peripheral T lymphocytes, certain agents such as catecholamines, prostaglandin E_1, and theophylline cause rather large increases in total cAMP content, inhibit mitosis, and do not appear to stimulate phosphorylation of cellular proteins. Phytohemagglutinin, however, stimulates mitosis, causes a small rise in cAMP content, and stimulates phosphorylation of a relatively large number of cellular proteins (45). Some of the membrane-bound proteins are phosphorylated in vitro following the addition of exogeneous N^6 monobutyryl cAMP (150). In contrast, wheat germ agglutinin causes larger increases in cAMP, inhibits mitosis, and diminishes phosphorylation (45). Parker (45) has postulated that the phosphorylation of the membrane proteins by specific lectins might modify the transport activity of the membranes in the early events associated with mitogenesis. He argues that only a small pool of cAMP is involved. Bloom, Wedner & Parker (152) have published immunocytochemical data which showed that the increase in cAMP is limited to areas near the plasma membrane when the cells are stimulated by phytohemagglutinin. A more diffuse cytoplasmic fluorescence is seen with prostaglandin E_1 and other stimuli that inhibit mitogenesis. Thus controlled phosphorylation of a discrete area of the cell would be associated with stimulation of mitogenesis. The immunofluorescence data emphasize that the response to phytohemagglutinin is highly localized. It is postulated that both the increase in adenylate cyclase activity and the response by cAMP-dependent protein kinase are compartmentalized. Phytohemagglutinin must be recognized by these specific compartments and result in limited protein phosphorylation which brings about the specific response, mitogenesis.

Interactions with Microtubules and Microfilaments

Cells contain two other diffuse but functional compartments with which the cyclic nucleotides might interact, the cytoskeleton and the contractile elements. The microtubules and microfilaments are the principal components of these two elements and the regulation of cell shape, motility, and internal structure seems to depend ultimately upon their coordinated function. Interesting data have been presented that implicate the cyclic nucleotides in these processes (153, 154).

Microtubules are relatively long structures that are made from the polymerization of a subunit, tubulin. The disaggregation of the microtubules with subsequent rearrangement was noted and may be important during certain processes (155).

Partially purified tubulin contains several proteins that are consistently observed with certain preparative techniques. One of these microtubule-associated proteins is phosphorylated by a cAMP-dependent endogenous protein kinase (156). A role for cGMP in microtubular function has also been postulated (154). The microfilaments are comprised of actin and cytoplasmic myosin. Additional regulatory proteins such as those found in skeletal and cardiac muscle may also be present. The function of these complexes is thought to be contractile in nature (157). The regulation of contraction may involve, at least in part, cyclic nucleotide–dependent processes that are analogous to those observed in cardiac muscle (158). The importance of these two systems makes the study of cyclic nucleotide regulation of coordinated microtubule and microfilament function an exciting undertaking. Again the approach to this interrelationship might include biochemical studies of purified preparations as well as dual immunofluorescent studies to simultaneously localize cyclic nucleotide and the microtubules and microfilaments.

CONCLUSION

The major premise in the field of cyclic nucleotide research is that hormones regulate the intracellular levels of cAMP and cGMP. These effectors, in turn, modulate physiologic processes. Both reason and methodology have led investigators to equate a rise in cyclic nucleotide concentration with a causal role in the alteration of a measurable biologic phenomenon. This review has attempted to marshal the available evidence that indicates that this is not always true. While the specific examples are few in number the concept of compartmentalization certainly merits further study. In addition, the concept has received support from the many publications that discuss the intracellular compartmentalization of the components of the cyclic nucleotide system.

To further speculate it seems likely that cAMP is a messenger that is produced by a perturbation of cell membranes. The biologic effect is produced by binding either to a local receptor unit (usually but perhaps not always a unit of protein kinase) or by penetration into the intracellular milieu. The demonstrated translocation of the catalytic unit of cAMP-dependent protein kinase offers another mobile biologic effector of cAMP action. The magnitude of the cAMP response to a stimulus may very well determine the intracellular location of the biologic response. In other words the high levels of free cAMP generated in some instances may be necessary to saturate receptors that reside far from the plasma membrane.

In contrast, guanylate cyclase is found in almost every compartment of the cell. Cyclic GMP production seems to be regulated by the local biochemical conditions which are undoubtedly modulated by the action of hormones. With such flexibility in the production of cGMP, it seems unlikely that biochemical machinery to translocate the action over long cellular distances need exist. Subtle changes in cGMP concentration in a defined area of the cell may be all that is required to alter processes. Reasoning teleologically, this postulated local control and action of cGMP might explain why the concentration of cGMP is much lower than that of cAMP.

The concept of regulation at precise intracellular loci seems eminently reasonable, but continued experimentation, novel approaches, and open minds will be necessary to further substantiate a hypothesis of compartmentalization of cyclic nucleotide action.

ACKNOWLEDGMENT

We would like to express our appreciation to Celeste Layton for her care in the preparation of this manuscript.

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