A Ca^{2+} -linked increase in coupled cAMP synthesis and hydrolysis is an early event in cholinergic and β -adrenergic stimulation of parotid secretion*

(phosphoryl labeling with ^{18}O /phosphodiesterase/adenylate cyclase/ionomycin)

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Communicated by Paul D. Boyer, June 27, 1988

ABSTRACT The dynamics and compartmental characteristics of cAMP metabolism were examined by ^{18}O labeling of cellular adenine nucleotide α phosphoryls in rat parotid gland stimulated to secrete with β -adrenergic and cholinergic agents. The secretory response occurred in association with a rapidly increased rate of cAMP hydrolysis apparently coordinated with an equivalent increase in the rate of cAMP synthesis, since the cellular concentration of cAMP remained unchanged. The magnitude of this metabolic response was equivalent to the metabolism of 10–75 times the cellular content of cAMP within the first minute of stimulation. This increased metabolic rate occurred only during the early (1–3 min) period of stimulation, in what appeared to be an exclusive cellular compartment distinguished by a unique distribution of ^{18}O among adenine nucleotide α phosphoryls. This ^{18}O distribution contrasted with that produced by forskolin, which increased cellular cAMP concentration and elicited only a delayed response missing the early secretory component. The early acceleration of cAMP metabolism appeared linked to a stimulus-induced increase in intracellular Ca^{2+} concentration, since the Ca^{2+} ionophore ionomycin produced the same metabolic response in association with secretion. These observations suggest that cAMP metabolism is involved in stimulus-secretion coupling by a Ca^{2+} -linked mechanism different from that in which cAMP plays the role of a second messenger.

The primary intracellular mediator of β -adrenergic-stimulated amylase release from the rat parotid gland is thought to be cAMP (1). However, in a number of reports, β -adrenergic stimulation of amylase release has been dissociated from any elevation of cellular cAMP concentration. For example, low concentrations of isoproterenol stimulate amylase release without detectable increases in total tissue cAMP content (2). The β -adrenergic agonists salbutamol and 1-(4-hydroxyphenyl)-2-isopropylaminoethanol (PI-39) stimulate amylase secretion without increasing cellular cAMP content at any concentration of either secretagogue (2, 3). Also, carbachol can promote amylase secretion without increasing cAMP levels (4) and can enhance secretion induced by submaximal concentrations of isoproterenol even though it partially suppresses the isoproterenol-induced elevation of cAMP concentration (5, 6).

An alternative to the classical second-messenger role that cAMP is well recognized to play is a more recently suggested involvement whereby the hydrolysis of the energy-rich cyclic nucleotides by certain species of phosphodiesterase is viewed to represent an energy-linked catalysis that subserves signal-transduction processes (7). This concept of the biological importance of cyclic nucleotides predicts that stimulus-induced increased rates of hydrolysis would be highly

coordinated with equivalent increased rates of cAMP synthesis with little or no change occurring in cellular concentration of the cyclic nucleotide. Support for this suggested “non-second-messenger” involvement of cyclic nucleotides has been obtained from studies of cGMP metabolism in vertebrate photoreceptors (8–10). To identify the occurrence of a cyclic nucleotide metabolic system of this type, a procedure has been developed to measure stimulus-induced changes in the dynamics of cyclic nucleotide metabolism in intact cells (11). The procedure involves determination of the rate of ^{18}O incorporation from [^{18}O]water into the phosphoryl of the 5'-nucleotide product. This ^{18}O insertion occurs upon phosphodiesterase-catalyzed hydrolytic cleavage of the cyclic nucleotide 3' cyclophosphoester bond. The ^{18}O incorporated into the phosphoryl of AMP is stoichiometric and nonexchangeable. Upon phosphorylation of [α - ^{18}O]AMP, which occurs rapidly in intact cells, the ^{18}O will also appear in the α phosphoryl of ADP ([α - ^{18}O]ADP) and ATP ([α - ^{18}O]ATP). To determine the total amount of cAMP hydrolyzed in a period of time, the content of ^{18}O in the α phosphoryls of all the adenine 5'-nucleotides must, therefore, be measured. In this report experiments are described in which this procedure was used to determine the changes in the dynamics of cAMP metabolism in rat parotid gland related to stimulus-secretion coupling.

MATERIALS AND METHODS

Parotid pieces were prepared from male Sprague-Dawley rats (175–225 g; Harlan; animal care was provided in accordance with University of Minnesota guidelines) fasted overnight but given water ad libitum. The 0.4-mm³ cubes of parotid tissue were prepared from glands obtained from anesthetized animals (Nembutal, 50 mg/kg, i.p.) and maintained at 37°C in Krebs-Ringer/Hepes solution (KRH: 128 mM NaCl/25 mM Hepes/4.7 mM KCl/2.5 mM CaCl_2 /1.2 mM MgSO_4 /1.2 mM Na_2PO_4 /5 mM D-glucose/5 mM sodium DL- β -hydroxybutyrate, pH 7.4 at 37°C) with an atmosphere of 100% O_2 . The tissue cubes were divided into portions (200–300 mg wet weight), transferred to tubes containing 600–750 μl of KRH, and preincubated for at least 30 min. Incubations were begun by addition of an equal volume of KRH (600–750 μl) prepared with [^{18}O]water (^{18}O -KRH); the final atom-percent enrichment of the medium ranged from 30% to 40%.

Abbreviations: PI-39, 1-(4-hydroxyphenyl)-2-isopropylaminoethanol; InsP_3 , inositol 1,4,5-trisphosphate; ^{18}O -KRH, Krebs-Ringer/Hepes solution made in [^{18}O]water.

*A preliminary report of this work was presented at the Sixty-Ninth Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 21–26, 1985 and published in abstract form (32).

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The incubation was terminated at the times designated by adding ice-cold 3 M perchloric acid, to a final concentration 0.5 M, and freezing the sample in liquid N₂. The acidified sample was thawed and then was sonicated three times for 10-sec durations while on ice to disrupt the cells. After centrifugation, the acidified extract was removed, neutralized with KHCO₃, and stored at -70°C until analysis for ¹⁸O enrichment of nucleotide phosphoryls and for nucleotide content was carried out (11, 12). Rates of cellular cAMP hydrolysis were determined from rates of appearance of ¹⁸O in the α phosphoryls of endogenous adenine nucleotides. The analytical procedure has been described in detail (11).

Tissue cAMP content was determined by radioimmunoassay (13) with antibody kindly provided by A. Steiner (University of Texas, Houston). Medium amylase content was assayed as described by Bernfeld (14). K⁺ release was monitored by removing 40–60 μ l of medium prior to adding the secretagogue and 1 sec before terminating the incubation. K⁺ content of the medium was determined by flame photometry. Changes in the medium K⁺ content represent net K⁺ efflux and were measured in incubations conducted in parallel with those in which ¹⁸O labeling was determined. Total cellular K⁺ was determined after sonication of parotid tissue.

Secretagogues were prepared in concentrated solutions and, upon addition to the incubation medium, were diluted 100-fold to achieve the concentrations designated. Isoproterenol, carbachol, and PI-39 were dissolved in water. Forskolin was dissolved in 95% ethanol, which, upon dilution in the medium, was less than 0.2% (vol/vol) ethanol, a concentration that does not increase cAMP concentration (15). PI-39 was a gift from J. Durham (Department of Biochemistry, West Virginia University, Morgantown).

RESULTS

¹⁸O Labeling of Endogenous Adenine 5'-Nucleotide α Phosphoryls Under Basal Conditions. In the basal state, the appearance of ¹⁸O in the phosphoryl of AMP and the α phosphoryls of ADP and ATP is linear for up to 45 min (data not shown). Adenine nucleotide species containing one, two, or three atoms of ¹⁸O in the α phosphoryls appear sequentially with time (data not shown) as observed in other types of cells (8, 12, 16). The basal rate of cAMP metabolic flux calculated from the rate of appearance of ¹⁸O in the α phosphoryls of the three adenine nucleotides was 628 ± 56 pmol per mg of protein per min (mean $\pm$ SEM, $n = 6$). This represents a basal cAMP metabolic flux that consumes the equivalent of 34% of the total cellular ATP concentration (3.7 nmol per mg of protein) per minute, taking into account that two molecules of ATP are required to regenerate each molecule of cAMP that becomes hydrolyzed.

Effects of Forskolin, PI-39, or Isoproterenol on cAMP Metabolism and Amylase Secretion. Forskolin (10 μ M), an agent that directly stimulates the catalytic subunit of cell plasma membrane-associated adenylyl cyclase (17), elevated the cellular concentration of cAMP 4.3-fold (Fig. 1B) within the first minute. In association with this increase in cAMP concentration, cAMP hydrolytic rate increased by 318 pmol per mg of protein per min. The secretory response to forskolin, unlike the increase in cAMP hydrolytic flux, was not immediate; no increase in amylase secretion was detectable in the first minute but by 3 min the rate of secretion was twice that of the control (Fig. 1B).

The forskolin-induced increase in cAMP hydrolytic rate was characterized by increases in the appearance of ¹⁸O primarily in the α phosphoryls of ATP (75%) and ADP (50%) with little increase in the ¹⁸O labeling of the phosphoryl of AMP (11%; Fig. 1A). This distribution of ¹⁸O primarily in α phosphoryls of the nucleoside di- and triphosphates closely resembles that observed when the concentration of cAMP is

FIG. 1. Comparison of the effects of forskolin, PI-39, and isoproterenol on the incorporation and distribution of ¹⁸O among the adenine 5'-nucleotide α phosphoryls, cAMP content, and amylase secretion. (A, C, and E) Effect of forskolin (10 μ M), PI-39 (1 μ M), or isoproterenol (1 μ M), respectively, on the ¹⁸O labeling of the adenine 5'-nucleotide α phosphoryls after 1 min of stimulation. Results are shown as percent increase over control. The secretagogue was added 1 min after the addition of ¹⁸O-KRH and the incubation was terminated 1 min later. (B, D, and F) Effect of forskolin, PI-39, or isoproterenol, respectively, on cAMP levels and amylase secretion as percent increase over the control value. The cAMP content under basal conditions was 3.7 ± 0.7 pmol per mg of protein (mean $\pm$ SEM, $n = 8$). The cAMP concentrations are those at 1 min after stimulation in the same tissues in which ¹⁸O-labeling rates were determined. Basal amylase secretion rate was 0.32 ± 0.07 units per mg of protein per min (mean $\pm$ SD, $n = 3$), where 1 unit equals 1 mg of maltose formed per 5 min. Values of ¹⁸O incorporation rate and cAMP concentration for isoproterenol and forskolin represent the average of triplicate determinations $\pm$ half the range from a single, representative experiment. Values for PI-39 are the average of two separate experiments $\pm$ half the range. Values for amylase secretion represent mean $\pm$ SEM of three separate experiments.

increased in platelets with prostacyclin (12) or when cGMP concentration is increased in neuroblastoma with sodium nitroprusside (16). This pattern of enhanced ¹⁸O appearance conforms to a previously proposed cellular metabolic model (12) whereby the greater amount of cAMP generated saturates the phosphodiesterase hydrolytic capability of the compartment in which basal cAMP metabolism occurs. An increased phosphodiesterase catalytic rate occurs under these circumstances because of the approach to saturation of

enzyme activity that was subsaturated under basal conditions. The cAMP not hydrolyzed, because it exceeds this phosphodiesterase catalytic capacity, may diffuse from the basal metabolic compartment to a larger cytoplasmic compartment. It was suggested that in this new locale, cAMP can subserve the function of a second messenger by interacting with an intracellular receptor such as a protein kinase and/or undergo hydrolysis to 5'-AMP that rapidly becomes phosphorylated to [α - ^{18}O]ATP (12). This [α - ^{18}O]ATP would become diluted by the much greater amount of unlabeled ATP in this larger compartment where the [α - ^{18}O]ATP would, therefore, accumulate. This metabolic model, which depends on increasing the mass of cAMP or cGMP within the cell, applies when cyclic nucleotides are intended to act as allosteric effectors.

Metabolic and secretory responses of the parotid to PI-39 were examined next. This secretagogue is structurally similar to isoproterenol except for the absence of the *meta* hydroxyl group [i.e., 1-(4-hydroxyphenyl)-2-isopropylaminoethanol] and is classified as a β -adrenergic agent (3, 18). It exhibits the feature of stimulating secretion without elevating the cellular cAMP concentration unless a phosphodiesterase inhibitor is present (3). Also relevant is that the activation of cAMP-dependent protein kinase is not detectable when PI-39 stimulates parotid secretion in the absence of a phosphodiesterase inhibitor, but is detectable when the inhibitor is present (3). In our experiments, PI-39 did not increase the cAMP level in the parotid at 1 min (Fig. 1D) or at times up to 4 min (data not shown). (The medium was not supplemented with a phosphodiesterase inhibitor.) PI-39 (1 μM) did, however, stimulate an $\approx$ 5-fold increase in amylase release within the first minute (Fig. 1D). This enhanced rate of amylase secretion returned almost to the control rate by 3 min (data not shown). Another effect of PI-39 was to increase the rate of cAMP hydrolysis by 45 pmol per mg of protein during the first minute of PI-39 stimulation. This PI-39-stimulated hydrolytic rate must have been accompanied by an equivalent increase in the rate of cAMP synthesis, because no change occurred in the cellular cAMP concentration even though this increased hydrolytic rate represented the consumption of >10 times the total amount of cAMP in the cell in no more than a minute.

The most striking difference between the PI-39- and forskolin-stimulated metabolism of cAMP, in addition to their contrasting effects on cellular cAMP levels, is the distribution of ^{18}O among the three adenine 5'-nucleotides. With PI-39, in contrast to forskolin, the phosphoryl of AMP was enriched the most in ^{18}O (40% increase), whereas the ATP α phosphoryl was labeled least (<5%; Fig. 1C). Although both forskolin and PI-39 stimulate amylase secretion and cAMP metabolic flux, there appear to be several differences in their actions. Unlike PI-39, which stimulates a rapid secretion that is not sustained, the effect of forskolin on secretion is not immediate (i.e., later than 1 min), although forskolin action to increase cAMP concentration and metabolic flux is relatively fast (i.e., within 1 min). In addition, the metabolic compartment in which cAMP metabolism is enhanced by forskolin most likely differs from that stimulated by PI-39, since the marked differences in ^{18}O distribution among the 5'-nucleotides are not otherwise explainable. The reason for the more prominent ^{18}O labeling of the phosphoryl of AMP by PI-39 stimulation is not known. However, this outcome would be consistent with cAMP undergoing metabolism in a cellular compartment in which the nucleotides undergoing the more rapid rate of metabolism exchange slowly or not at all with nucleotides in larger compartments.

The effects produced by isoproterenol (1 μM), a β -adrenergic agent that elevates cellular cAMP concentration, resembled a composite of those observed with forskolin and PI-39. Isoproterenol increased the concentration of cAMP

>8-fold (Fig. 1F), the metabolic flux of cAMP by 566 pmol per mg of protein per min, and the secretion of amylase by about 4-fold during the first minute (Fig. 1F). The ^{18}O distribution in the phosphoryls of the three adenine 5'-nucleotides showed that ATP became enriched with ^{18}O by an additional 60%, while relatively large increases in ^{18}O labeling also occurred in AMP (132%) and α -ADP (176%; Fig. 1E).

These results indicate no correlation between the rapid secretory response and increases in cellular cAMP concentration. On the other hand, there is a correlation between the rapid secretory response and the stimulus-induced acceleration of cAMP metabolic rate, if the cAMP metabolic response considered is represented by that resulting in the ^{18}O labeling of a relatively large proportion of the cellular AMP.

Effect of Carbachol on cAMP Metabolism and K^+ Secretion.

In the rat parotid gland, muscarinic receptor activation stimulates amylase secretion, but the more prominent effect is to enhance K^+ efflux (4). When K^+ efflux was measured to monitor the cellular response, 1 and 10 μM carbachol stimulated net efflux of this cation incrementally (Fig. 2B). This muscarinic-stimulated response was associated with no detectable change in cAMP concentration [$102 \pm 6\%$ (mean $\pm$ range, $n = 2$) and $92 \pm 6\%$ (mean $\pm$ SEM, $n = 3$) of control with 1 and 10 μM carbachol, respectively]. The absence of any effect of muscarinic stimulation on basal cellular cAMP concentration has been reported (4). However, from the rate of appearance of ^{18}O in adenine 5'-nucleotide α phosphoryls, it was apparent that the cAMP hydrolytic rate was increased within 1 min by 37 ± 5 (mean $\pm$ range, $n = 2$) and 265 ± 58 (mean $\pm$ SEM, $n = 3$) pmol per mg of protein with 1 and 10 μM carbachol, respectively. This higher rate of cAMP hydrolysis with 10 μM carbachol represents the equivalent of 75 times the total cellular content of cAMP hydrolyzed per minute. Since the cellular cAMP concentration is maintained under these circumstances, muscarinic stimulation must have effected an increase in adenylyl cyclase activity equivalent in magnitude to the increase in activity of cAMP phosphodiesterase(s).

Of the three adenine 5'-nucleotide α phosphoryls, AMP labeling with ^{18}O was increased to the greatest extent with carbachol. A smaller increase was seen in the ^{18}O labeling of the α phosphoryl of ADP, and there was an apparent decrease in the total ^{18}O present in the α phosphoryl of ATP (Fig. 2A). This decrease in α -ATP labeling with ^{18}O resulted from 10% and 20% decreases (data not shown) in the cellular concentration of ATP accounted for by increases in the levels of ADP and AMP with 1 and 10 μM carbachol, respectively. Cholinergic stimulation of the parotid has been reported to

FIG. 2. Effects of 1 and 10 μM carbachol on ^{18}O labeling of the α phosphoryls of adenine 5'-nucleotides (A) and net K^+ efflux (B). For 1 μM carbachol, values represent the mean of two separate experiments $\pm$ half the range. Values for 10 μM carbachol represent the mean $\pm$ SEM for three separate experiments.

decrease ATP concentration in the parotid (19). Labeling with ^{18}O of AMP and the α phosphoryl of ADP increased 195% and 125%, respectively, with 1 μM carbachol, whereas 10 μM carbachol increased AMP and ADP labeling by 445% and 250%, respectively (Fig. 2A).

Since carbachol stimulation alters the levels of adenine nucleotides, a fraction of the increased ^{18}O labeling of AMP and ADP might be viewed as having derived from the $[\alpha\text{-}^{18}\text{O}]\text{ATP}$ that was degraded. With PI-39 stimulation, a similar distribution of ^{18}O predominantly in AMP occurred independent of net ATP degradation. From consideration of the characteristics of nucleotide phosphoryl labeling with ^{18}O , the degraded ATP would have contributed very little to this relatively large increase in ^{18}O labeling of AMP.

On the other hand, it is very likely that a portion of the $[\alpha\text{-}^{18}\text{O}]\text{ATP}$ recycles within a discrete metabolic compartment to participate in one or more additional rounds of cAMP synthesis. This would result in the incorporation of a second atom of ^{18}O into AMP when these molecules of cAMP become hydrolyzed. Experimental evidence supporting metabolic recycling has been obtained from the detection of AMP molecules that contain two atoms of phosphoryl ^{18}O . This multiple labeling of AMP with ^{18}O is much more prominent after cholinergic and PI-39 stimulation. This is clearly demonstrated by the ratio of doubly to singly ^{18}O -labeled molecules ($^{18}\text{O}_2/^{18}\text{O}_1$) of AMP, which was found to be 1:5.8 after carbachol stimulation compared to 1:11.5 in nonstimulated controls after the first minute of incubation in ^{18}O water. A similarly low $^{18}\text{O}_2/^{18}\text{O}_1$ ratio in AMP was found after PI-39 stimulation (e.g., 1:6.1) but not when forskolin was the stimulus (e.g., 1:10.1); isoproterenol produced an $^{18}\text{O}_2/^{18}\text{O}_1$ ratio (1:8.7) midway between that of forskolin and PI-39. These results indicate that the metabolic pool of cAMP and related adenine 5'-nucleotides involved in the metabolic response to muscarinic stimulation, and probably to a component of β -adrenergic stimulation, are distinct from those brought into play by stimuli that elevate cAMP concentration.

The time course of carbachol (10 μM)-stimulated cAMP metabolic flux was determined by pulse additions of ^{18}O water to monitor the rate of ^{18}O incorporation during intervals after introduction of the stimulus (data not shown). Accelerated cAMP metabolism induced by carbachol was maximal during the first minute after addition of carbachol, declined to half the maximal rate by 1.5 min, and was nearly at the nonstimulated rate by 2 min. This time course of enhanced cAMP metabolic rate very closely coincided with the initial phase of K^+ (and probably amylase) efflux, which was also maximal at 1 min and almost at the nonstimulated level by 3 min (data not shown and ref. 20).

Effect of Ionomycin on cAMP Metabolism and K^+ Secretion. The carbachol- and PI-39-stimulated rates of cAMP metabolism most probably involve simultaneous stimulation of species of adenylyl cyclase and phosphodiesterase, since no change in the cellular cAMP level is detectable even when 10–75 times the total amount of cAMP in the cell undergoes metabolism. Cholinergic and β -adrenergic stimuli are generally thought to have opposing effects on adenylyl cyclase activity; the latter stimulatory, acting through a G_s species of guanine nucleotide-binding regulatory protein (21), and the former inhibitory, acting through a G_i species (21, 22). However, a common feature of both classes of stimuli in secretory cells is a rapid increase in intracellular Ca^{2+} concentration. Cholinergic stimuli effect this Ca^{2+} transient by a rapid generation of inositol 1,4,5-trisphosphate (InsP_3) (23), whereas β -adrenergic agents do so by an undefined mechanism (24–26) that does not involve InsP_3 (27) and that is independent of increases in cellular cAMP concentration (28). Also, since there are species of Ca^{2+} /calmodulin-stimulable adenylyl cyclase (29) and phosphodiesterase (30)

in parotid cells, it is possible that intracellularly released Ca^{2+} could underlie the stimulated rates of cAMP metabolism by these diverse classes of stimuli. This possibility was explored by examining the effect of the Ca^{2+} ionophore ionomycin on cAMP metabolism. Ionomycin (10 μM) increased cAMP hydrolytic flux by 37 pmol per mg of protein per min without affecting the cellular cAMP concentration (103% of control; data not shown). Net K^+ efflux during the first minute of incubation with 10 μM ionomycin was 20 nmol per mg of protein per min, a rate comparable to that of 2–4 μM carbachol (data not shown). The additional atoms of ^{18}O appearing in the α phosphoryl of the adenine 5'-nucleotides with ionomycin-stimulated cAMP hydrolytic rate were selectively distributed, as with carbachol and PI-39, in AMP (45%) and ADP (34%) (compare Figs. 2A and 3). The total ^{18}O labeling of the α phosphoryl of ATP decreased 3%, with ionomycin stimulation, in association with a decrease of 10% in ATP concentration (data not shown). These results indicate that increasing intracellular Ca^{2+} concentration can bring about an increase in cAMP metabolic flux that, in several respects, is characteristic of the effect produced by carbachol and by the β -adrenergic agent PI-39; all three elicited this metabolic response without any detectable alteration in cellular cAMP concentration.

DISCUSSION

These results demonstrate that an early response of parotid cells to both cholinergic and β -adrenergic agonists is an increased rate of cAMP metabolism that does not result in any detectable change in cellular cAMP concentration. This observation would not be predicted from classical cholinergic or β -adrenergic action, since the former is ordinarily thought to decrease, and the latter to increase, cellular cAMP concentration. Paradoxically, the two types of agents have similar qualitative effects on parotid secretion. The view provided by these results is that both classes of stimuli have a common effect of stimulating a cAMP metabolic system in which the rate of cAMP metabolism, not the concentration of cAMP, is increased. This cAMP metabolic system, which requires monitoring of the dynamics of cellular cAMP metabolism to detect, appears to be distinct from the system metabolizing the cAMP that serves the role of regulating cell function as a second messenger. In addition to the novel characteristic of operating without altering the concentration of cellular cAMP, this cAMP metabolic system is very rapidly activated for only about 2 min (e.g., by a cholinergic stimulus) and appears to function in a discrete cellular locale distinguishable from that in which second-messenger metab-

FIG. 3. Effect of ionomycin (10 μM) on the incorporation and distribution of ^{18}O among adenine nucleotide α phosphoryls. Values are shown as percent of ^{18}O incorporation above control and represent the average of duplicate determinations from a single experiment $\pm$ half the range.

olism of cAMP occurs. It also seems to exhibit the novel feature of being activated by Ca^{2+} released by these stimuli rather than by the more direct mechanism involving a membrane receptor-mediated stimulation of adenylyl cyclase of the second-messenger metabolic system.

The mechanism that provides for such apparently fine coordination between cAMP synthesis and hydrolysis remains to be elucidated. The occurrence in parotid of species of adenylyl cyclase and phosphodiesterase sensitive to Ca^{2+} /calmodulin could be construed to meet such a demand, but this is only one of several possibilities (29, 30). On the other hand, the demonstrated effect of ionomycin to increase the rate of cAMP metabolism and the temporal discreteness of the carbachol-induced increase in cAMP metabolism to approximate so closely the carbachol-stimulated generation of InsP_3 (27) and release of intracellular Ca^{2+} in parotid (31) strengthen the conclusion that Ca^{2+} serves as the trigger for this cAMP metabolic response.

In the context of the two recognized phases (i.e., rapid and sustained) of cholinergic- and β -adrenergic-stimulated secretion, it seems that the intracellularly released Ca^{2+} that appears to accelerate cAMP metabolism without increasing cAMP concentration is associated with the rapid phase. The delayed, sustained phase of secretion (seen with forskolin) may be related to the prolonged elevation of cAMP concentration and, in the case of cholinergic stimulation, perhaps the secondary influx of extracellular Ca^{2+} (20). Increased concentrations of cAMP or Ca^{2+} associated with delayed, prolonged secretion would have the capability of acting similarly; both cAMP and Ca^{2+} activate species of protein kinases.

The mechanism by which stimulus-induced increases in cAMP metabolic rate may be of functional utility in stimulus-secretion coupling is not definable at this juncture. A similar cyclic nucleotide metabolic system in which cGMP undergoes rapid synthesis and hydrolysis has been identified in vertebrate photoreceptors (8–10). Possible mechanisms by which this metabolic unit may be of utility in phototransduction have been offered (8), but none is yet established. Even a speculative model regarding its possible involvement in secretion is constrained by the lack of biochemical definition of the secretory process. However, our working model is based on the phosphodiesterase-catalyzed hydrolytic cleavage of the energy-rich 3' bond of the cyclic nucleotide participating in an energy-linked process that effects a conformational change of a critical secretory component.

The skillful GC/MS analysis of phosphoryl ^{18}O by Eric Butz is gratefully acknowledged. M.A.D. is the recipient of a Medical Scientist Scholarship from the Life and Health Insurance Medical Research Fund. This work was supported by the National Institutes of Health (GM28818, EY04877, DK38481), the Minnesota Medical Foundation, and the Minnesota Leukemia Research Fund. This paper was submitted in partial fulfillment of Ph.D. thesis work (M.A.D.) at the University of Minnesota.

- Butcher, F. R. & Putney, J. W., Jr. (1980) *Adv. Cyclic Nucleotide Res.* **13**, 215–249.
- Butcher, F. R., Goldman, J. A. & Nemerovski, M. (1975) *Biochim. Biophys. Acta* **392**, 82–94.
- Spearman, T. N., Durham, J. P. & Butcher, F. R. (1982) *J. Cyclic Nucleotide Res.* **8**, 225–234.
- Butcher, F. R., McBride, P. A. & Rudich, L. (1976) *Mol. Cell. Endocrinol.* **5**, 243–254.
- Harper, J. F. & Brooker, G. (1977) *Mol. Pharmacol.* **14**, 1031–1045.
- Oron, Y., Kellogg, J. & Lerner, J. (1978) *Mol. Pharmacol.* **14**, 1018–1030.
- Goldberg, N. D. & Haddox, M. K. (1977) *Annu. Rev. Biochem.* **46**, 823–896.
- Goldberg, N. D., Ames, A., III, Gander, J. E. & Walseth, T. F. (1983) *J. Biol. Chem.* **258**, 9213–9219.
- Ames, A., III, Walseth, T. F., Heyman, R. A., Barad, M., Graeff, R. M. & Goldberg, N. D. (1986) *J. Biol. Chem.* **261**, 13034–13042.
- Dawis, S. M., Graeff, R. M., Heyman, R. A., Walseth, T. F. & Goldberg, N. D. (1988) *J. Biol. Chem.* **263**, 8771–8785.
- Walseth, T. F., Graeff, R. M. & Goldberg, N. D. (1988) *Methods Enzymol.* **159**, 60–74.
- Walseth, T. F., Gander, J. E., Eide, S. J., Krick, T. P. & Goldberg, N. D. (1983) *J. Biol. Chem.* **258**, 1544–1558.
- Zeilig, C. E. & Goldberg, N. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1052–1056.
- Bernfeld, P. (1955) *Methods Enzymol.* **1**, 149–153.
- Harper, J. F. & Brooker, G. (1980) *J. Cyclic Nucleotide Res.* **6**, 51–62.
- Graeff, R. M., Walseth, T. F. & Goldberg, N. D. (1987) *Neurochem. Res.* **12**, 551–560.
- Seamon, K. & Wetzel, J. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 91–99.
- Bilezikian, J. P., Dornfeld, A. M. & Gammon, D. E. (1978) *Biochem. Pharmacol.* **27**, 1445–1454.
- Kangasunpheram, P. & Lim, S. C. (1978) *Biochem. J.* **176**, 23–29.
- Putney, J. W., Jr. (1976) *J. Pharmacol. Exp. Ther.* **198**, 375–384.
- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649.
- Watanabe, A. M., McConaughy, M. M., Strawbridge, R. A., Fleming, J. W., Jones, L. R. & Besch, H. R. (1978) *J. Biol. Chem.* **253**, 4833–4836.
- Kikuchi, A., Kozawa, B., Kaibuchi, K., Katada, T., Ui, M. & Takai, Y. (1986) *J. Biol. Chem.* **261**, 11558–11562.
- Spearman, T. N. & Butcher, F. R. (1984) in *The Role of Calcium in Biological Systems*, eds. Anghileri, L. J. & Tuffet-Anghileri, A. M. (CRC, Boca Raton, FL), Vol. 1, pp. 122–146.
- Takemura, H. (1985) *Biochem. Biophys. Res. Commun.* **131**, 1048–1055.
- Butcher, F. R. (1980) *Biochim. Biophys. Acta* **630**, 254–260.
- Aub, D. L. & Putney, J. W., Jr. (1985) *Biochem. J.* **225**, 263–266.
- Dreux, C., Imhoff, V., Hyleux, C., Busson, S. & Rossingnol, B. (1986) *Am. J. Physiol.* **251**, C754–C762.
- Piascik, M. T., Babich, M., Jacobson, K. L. & Watson, E. L. (1986) *Am. J. Physiol.* **250**, C642–C645.
- Ku, K. Y. & Butcher, F. R. (1980) *Biochim. Biophys. Acta* **631**, 70–78.
- Merritt, J. E. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 4958–4960.
- Deeg, M. A., Walseth, T. F., Graeff, R. M. & Goldberg, N. D. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 697 (abstr.).