Proc. Nat. Acad. Sci. USA
Vol. 73, No. 1, pp. 156–160, January 1976
Cell Biology

Cellular cyclic nucleotides and enzyme secretion in the pancreatic acinar cell*

(3′:5′-cyclic AMP/3′:5′-cyclic GMP/stimulus secretion coupling/pancreatic secretion)

ASHER HAYMOVITS AND GEORGE A. SCHEELE

The Rockefeller University, New York, NY 10021

Communicated by Philip Siekevitz, October 9, 1975

ABSTRACT **Cellular levels of cAMP and cGMP were measured in guinea pig pancreatic lobules incubated *in vitro*, during basal or stimulated secretion. Stimulation with optimal concentrations of carbamylcholine (carbachol) (10^{-5} M), pancreozymin (0.1 unit/ml), and caerulein (10^{-9} M) resulted within seconds in a sharp rise in cGMP levels, from five to more than 20 times that of basal levels. cAMP levels did not change significantly. cGMP increases were maximal at 2 min then subsided by 4–7 min to a plateau about two to three times that of basal level. This plateau was maintained for the duration of the secretagogue stimulus. Removal of the carbachol stimulus resulted in a rapid decrease in cGMP levels to that of the basal state. The cellular cGMP levels observed within the first 2 min of stimulation correlated closely with the dose of carbachol and the secretory response. Atropine at 10^{-4} M blocked the cGMP elevation due to carbachol but not that due to pancreozymin, while carbonyl cyanide *m*-chlorophenyl hydrazone, an uncoupler of oxidative phosphorylation, blocked the response to both secretagogues. Similar though less extensive findings were observed using rabbit pancreatic lobules incubated *in vitro*. High concentrations (10^{-2}–10^{-3} M) of the dibutyryl and 8-bromo analogues of both nucleotides were effective, though suboptimal, secretagogues. In the case of the cAMP analogues, the secretory response was associated with a rise in endogenous cGMP levels, similar to that observed during suboptimal carbachol stimulation.**

These findings suggest that cGMP may be an intracellular mediator in the process of stimulus secretion coupling in the acinar cell of the exocrine pancreas.

Earlier attempts to define a role for cAMP in stimulus-secretion coupling in the acinar cell of the exocrine pancreas resulted in inconsistent findings (1–8). Recent reports of increased cellular levels of cGMP seen with cholinergic stimulation of the muscarinic type in various tissues (9–13) suggested that the acinar cell, also responsive to muscarinic stimulation, might have cGMP as an intracellular mediator. The availability of highly sensitive and specific radioimmunoassays for cGMP and cAMP (14) has enabled us to examine directly this possibility in a system of pancreatic lobules incubated *in vitro*, shown previously to be a reproducible and sensitive model for enzyme and zymogen secretion from the exocrine pancreas (15). Lobules of this type proved to be suitable for rapid and accurate measurement of cellular cAMP and cGMP in the same tissue specimens (16).

MATERIALS AND METHODS

Chemical Preparations. Carbamylcholine (carbachol) and L-[4,5-^{3}H]leucine were obtained from Schwarz-Mann Biochemicals, Orangeburg, N.Y. cGMP, N^6,$O^{2'}$-dibutyryl cGMP, N^6,$O^{2'}$-dibutyryl cAMP, 3′:5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.16), theophylline, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), and atropine sulfate were all from Sigma Chemical Co., St. Louis, Mo. 8-Bromo cAMP was from Plenum Scientific Co., N.Y., and 8-bromo cGMP from ICN Corp., Irvine, Calif. Purified pancreozymin was kindly supplied by Dr. V. Mutt, Karolinska Institutet, Uppsala, Sweden, and synthetic caerulein was a gift from Dr. B. Camerino, Farmitalia, Milan, Italy. Dowex resins were from Bio-Rad Inc., Richmond, Calif., and QAE Sephadex from Pharmacia Inc., Piscataway, N.J.

Pancreatic Lobule Preparations. Albino guinea pigs, 500 g in weight, and fed ad lib., were sacrificed by a blow to the head. The excised pancreas was immersed in cold Krebs-Ringer bicarbonate buffer, pH 7.4, equilibrated with 95% O_2–5% CO_2. Lobules were prepared as described (15). Each experimental flask contained 10 lobules (about 60 mg wet weight) supported on a nylon mesh disc (about 2 cm in diameter) in 5 ml of Krebs-Ringer buffer supplemented by physiological levels of glucose and amino acids. Secretagogues and other agents were added or omitted as indicated. Incubation was carried out in a shaker bath at 37° for varying periods of time under a 95% O_2–5% CO_2 atmosphere. Timed incubation was terminated by removal of the nylon mesh with the adherent lobules and instant freezing of the tissue in liquid nitrogen. Similar *in vitro* preparations of pancreatic lobules were made from pancreata excised from albino New Zealand rabbits 2.5 kg in weight; albino rats, 150–200 g; and mice, 25–30 g, all fed ad lib.

Extraction and Measurement of Cyclic Nucleotides. The frozen tissue was suspended and homogenized in 3 ml of ice-cold 6% trichloroacetic acid. The homogenate was centrifuged at 5000 × *g* for 15 min; the precipitate was used for the determination of DNA content by the diphenylamine method of Burton (17). The supernatant was extracted three times, each with 2 volumes of distilled water-saturated ether. The residue of the lyophilized aqueous phase was dissolved in 1.0 ml of 0.05 M acetate buffer, pH 6.2, and used for radioimmunoassay or further purification on a column of 0.7 × 3 cm of Bio-Rad AG-1-X8, 200–400 mesh, equilibrated with 0.1 M formic acid. After washing with 0.1 M formic acid, cAMP and cGMP were eluted with 1.0 M and 4.0 M formic acid, respectively, and the eluates were lyophilized. The residues were dissolved each in 1.0 ml of 0.05 M acetate buffer, pH 6.2, and aliquots were used for radioimmunoassay. In experiments where nucleotides were measured in the medium, the total medium was collected, frozen, acidified in 6% trichloroacetic acid, and processed as described above. When high concentrations of exogenous cAMP derivatives were used, the respective nucleotide fractions were further purified on a QAE-Sephadex A-25 column of 0.9 × 12 cm, eluted with ammonium formate (14). Specific radioimmunoassay of each of the separated nucleo-

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

* Presented in part before the 4th Annual Meeting of the American Pancreatic Study Group, October 31, 1974, Chicago, Ill. and the 59th Annual Meeting of the Federation of American Societies for Experimental Biology, April 17, 1975, Atlantic City, N.J.

tide fractions thus enabled minimal crossreactivity interference. The cGMP antiserum used discriminated against cross-reacting antigens in a ratio of 1 to 1000 or better.

Beef heart cyclic nucleotide phosphodiesterase was used to treat residues of control pancreatic lobules to assess the nucleotide specificity of the immunoassayable material in a manner as described by Steiner *et al.* (14).

^{125}I-Labeled derivatives and specific high-titer antisera for cyclic AMP and cyclic GMP were purchased from Collaborative Research Inc., Waltham, Mass. An in-house antiserum against a 2′-*O*-succinyl cGMP-hemocyanin conjugate was developed in rabbits. Antiserum with the ultimate 50% binding titer for labeled cGMP of >1:20,000 was used in the radioimmunoassay.

The procedure of radioimmunoassay for each nucleotide followed that reported by Steiner *et al.* (14) except that separation of bound from free derivative was routinely accomplished by precipitation in the cold with 60% saturated ammonium sulfate solution. An effective measurement range of 0.02–5.0 and 0.05–10.0 pmol was routinely observed for cAMP and cGMP, respectively, with virtually no crossreactivity. Recovery experiments with exogenously added pure nucleotides and labeled derivatives to homogenized lobule preparations yielded values ranging from 85 to 103% for cAMP and 80 to 95% for cGMP.

Tissue nucleotide levels were expressed as pmol/μg of DNA per sample. The nucleotide levels in the medium were similarly expressed, the DNA being derived from the tissue that was incubated in that medium.

Secretion Experiments. Flasks containing 8 to 10 lobules were used to monitor the effects of exogenous cAMP, cGMP, and their N^6,O^2-dibutyryl and 8-bromo substituted derivatives, and the phosphodiesterase inhibitors, theophylline and imidazole, on the secretory process in the resting or suboptimally stimulated (carbachol 10^{-6} or 10^{-7} M) state.

The lobules were pulse-labeled with 0.44 μM L-[4,5-^{3}H]leucine (20 μCi/ml) for 5 min, washed, transferred, and incubated in a second flask containing L-[^{1}H]leucine (2 mM) for 90 min, during which time the majority of the radioactive proteins were chased into the zymogen granule pool. The lobules were then transferred to the experimental flask, which contained additional chase medium (1–5 ml) with the nucleotide derivatives and inhibitors as indicated. Sequential aliquots of the medium withdrawn during the incubation and the homogenate of the tissue at the end of incubation (100-μl aliquots contained 250–4000 cpm) were processed for trichloroacetic acid-insoluble radioactivity (18, 19). The data were expressed as cumulative discharge of labeled protein into the incubation medium as percent of total labeled protein (tissue and medium) as a function of time. In critical experiments, cyclic nucleotide levels and secretion rates were measured in lobules taken from the same pancreatic gland and handled in an identical (parallel) manner.

RESULTS

Nucleotide levels in resting lobules

The cellular levels of cAMP and cGMP in nonstimulated lobules incubated *in vitro* for periods up to 120 min were remarkably constant. cAMP ranged between 0.1 and 0.4 pmol/μg of DNA. Similar values were reported for the liver (assuming: 1 μg of DNA $\cong$ 30 μg of protein $\cong$ 360 μg wet weight) using either radioimmunoassay (0.33 pmol/μg of DNA) (20) or enzymatic methods (0.4 pmol/μg of DNA) (21, 22). Lower values were reported earlier for cat pancreas using less specific enzymatic methods (1). Cyclic GMP levels were lower, ranging between 0.02 and 0.1 pmol/μg of DNA, and compared closely to levels reported for other tissues using radioimmunoassay (20). Neither cyclic nucleotide could be detected in the incubation medium of resting lobules even after prolonged incubation (120 min).

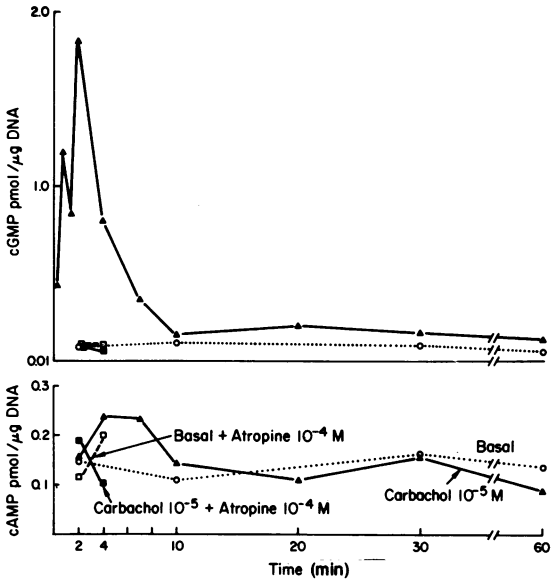

FIG. 1. Time course plot in minutes of cellular cGMP and cAMP levels in carbachol (10^{-5} M)-stimulated guinea pig pancreatic lobules in the presence or absence of atropine (10^{-4} M).

Response to carbachol stimulation

Carbachol (10^{-5} M) caused a rise in cellular cGMP levels, detected as early as 10 sec, which reached a maximum of 0.3 to > 1.0 pmol/μg of DNA (5 to >25 times that of basal levels) at 1–2 min (Fig. 1). Maximal levels occurred either as a single or a double spike. Subsequently, cGMP levels decreased to a plateau, approximately two to three times the basal level, which was maintained for the duration of the carbachol stimulus, up to 60 min (Fig. 1). Coincidentally, the nucleotide appeared in the medium. The cumulative total at the end of 10 min of stimulation did not exceed 0.1 pmol/μg of DNA. It should be pointed out, however, that because of the lack of degradative enzymes in the medium it is difficult to compare cGMP levels in the tissue with those in the medium. In contrast, optimal stimulation with carbachol did not significantly alter levels of cAMP in the tissue or in the medium (Fig. 1).

Effect of a muscarinic block and uncoupling of oxidative phosphorylation on the nucleotide response

Preincubation of pancreatic lobules for 10 min at 37° in the presence of atropine, a muscarinic blocking agent, at 10^{-4} M completely blocked the carbachol-induced rise in cellular cGMP (Fig. 1). Similarly, preincubation in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an agent that uncouples oxidative phosphorylation, at 10^{-4} M also prevented the cGMP response to carbachol (not shown). Atropine and CCCP have been shown to block the secretory response of pancreatic lobules to carbachol stimulation (15).

Effect of termination of stimulus

Termination of carbachol stimulation at the peak response of cellular cGMP (2-min time point) was effected by rapid transfer of the tissue to a second flask with medium lacking carbachol. Cellular cGMP levels subsided within 2–4 min to

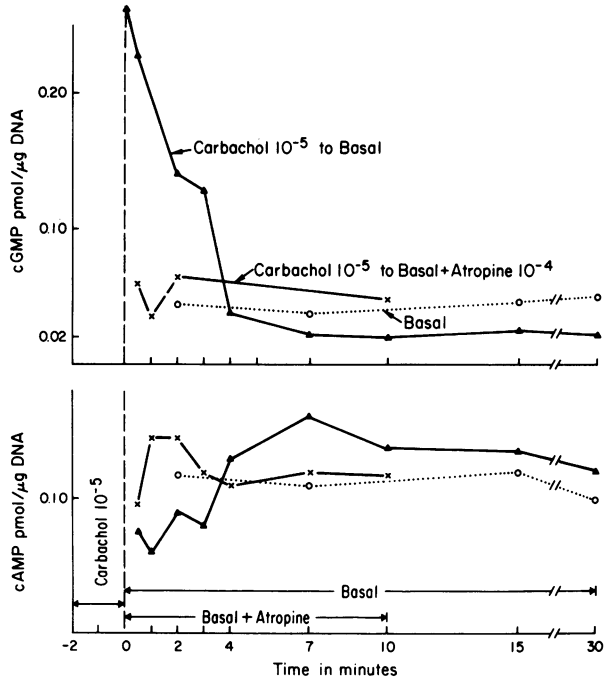

FIG. 2. The effect of termination of carbachol (10^{-5} M) stimulation on cellular cGMP and cAMP levels in guinea pig pancreatic lobules. Transfer to basal medium is compared to transfer to basal medium containing atropine (10^{-4} M).

the basal level (Fig. 2), as did the secretory response (not shown). Furthermore, recovery to basal levels occurred instantaneously in transfer medium with atropine (10^{-4} M). Similar return to basal levels was observed upon termination of stimulus after 30 min and 60 min of incubation.

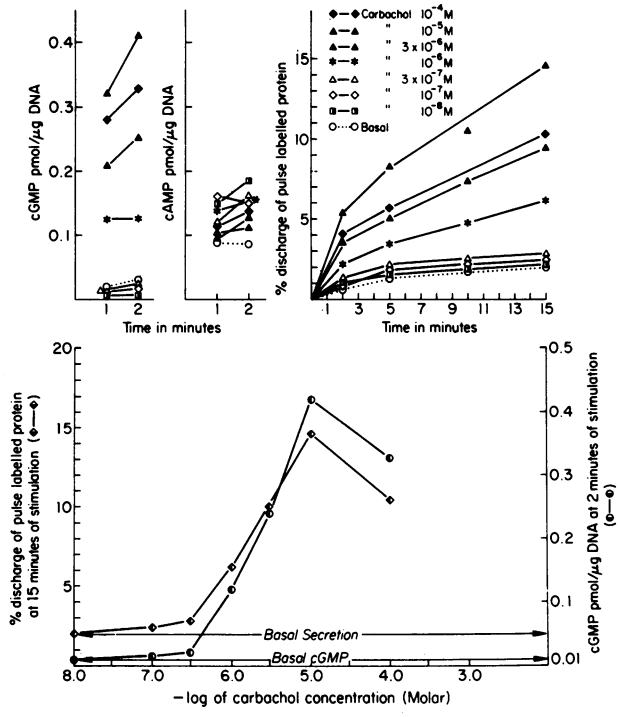

FIG. 3. Correlation of the cellular cGMP levels observed in the first 2 min of stimulation with the secretory response at 2–15 min over a concentration range of 10^{-8}–10^{-4} M carbachol. Upper panel from left: cGMP levels, cAMP levels, and secretory response. Lower panel: Comparison of cGMP response to the secretory response.

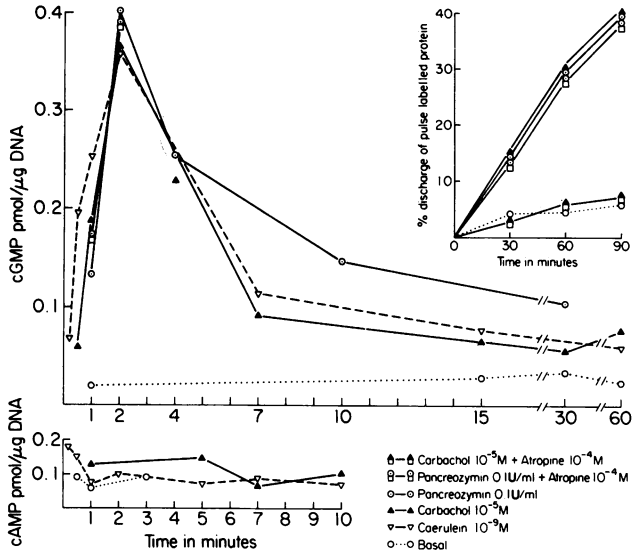

FIG. 4. Comparison of effects of carbachol, pancreozymin, and caerulein stimulation on cyclic nucleotides in guinea pig pancreatic lobules.

Dose-related response

The response in cGMP levels to carbachol stimulation peaked generally at the 2-min time point. The height of the peak observed was related to the dose of carbachol in the concentration range of 10^{-8}–10^{-4} M, with an optimum at 10^{-5} M (Fig. 3, upper panel, left). This was not observed for cAMP (Fig. 3, upper panel, middle). In two experiments where secretory response and cGMP levels were measured in lobules taken from the same pancreas and handled in an identical manner, initial rise in cGMP levels (2-min time point) correlated closely with initial secretory response, measured at 2- to 15-min intervals (Fig. 3, upper right and lower panels).

Effects of pancreozymin and caerulein stimulation

Incubation of pancreatic lobules in the presence of 10^{-9} M caerulein (a decapeptide amide analogue of the physiological pancreatic secretagogue, pancreozymin) or 0.1 unit/ml of pancreozymin, concentrations optimal for stimulation of enzyme secretion, caused an increase in cellular cGMP levels similar to that observed with optimal doses of carbachol. Again, no consistent changes in cellular cAMP levels were observed (Fig. 4, left upper and lower panels). Whereas at-

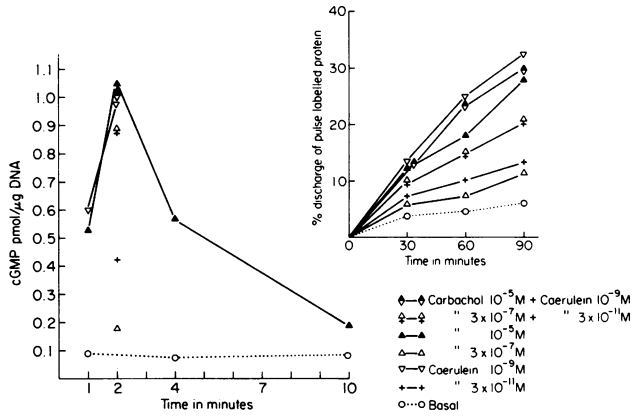

FIG. 5. Additive effect of suboptimal and optimal concentrations of carbachol and caerulein on cGMP and the secretory response in guinea pig pancreatic lobules.

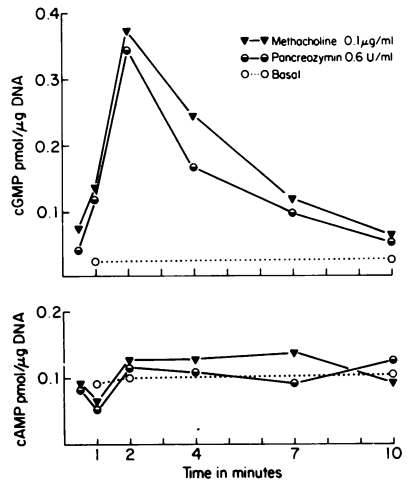

FIG. 6. Cyclic nucleotide levels in rabbit pancreatic lobules stimulated with optimal concentrations of methacholine and pancreozymin.

ropine at 10^{-4} M blocked the secretory as well as the cGMP effect of carbachol (Figs. 1 and 4), no such blocade was observed with pancreozymin stimulation (Fig. 4, upper left and right panels).

Additive effects of suboptimal carbachol and caerulein stimulation

The cellular cGMP response, as well as secretion of pancreatic lobules stimulated suboptimally by 3×10^{-7} M carbachol, were enhanced by the addition of the suboptimal stimulus, 3×10^{-11} M caerulein. In contrast, no enhancement of either cGMP level or secretion was observed when lobules were incubated in presence of optimal concentrations of the two secretagogues, 10^{-5} M carbachol and 10^{-9} M caerulein (Fig. 5).

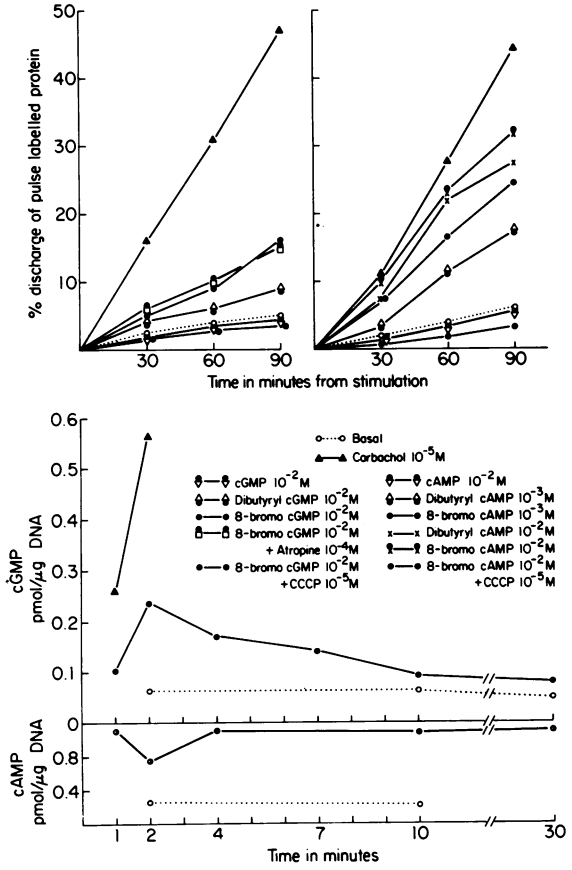

FIG. 7. Effects of exogenous cyclic nucleotide derivatives on guinea pig pancreatic lobule preparations. Upper panel: effects on secretion. Lower panel: effect of exogenous 8-bromo cAMP (10^{-3} M) on endogenous cGMP.

Cellular cGMP response in the pancreatic acinar cell of other species

Although the data reported above were derived from guinea pig pancreatic lobules, similar results were obtained in pancreatic lobules from other species. Rabbit pancreatic lobules responded to optimal methacholine stimulation (0.1 μg/ml) as well as to optimal pancreozymin stimulation (0.6 unit/ml) with a phasic change in cellular cGMP levels indistinguishable from that of the guinea pig. Here again, no significant changes were observed in cellular cAMP levels (Fig. 6). Similar, but much less pronounced and consistent responses were observed in rat and mouse pancreatic lobules.

Effect of phosphodiesterase inhibitors

Theophylline, a phosphodiesterase inhibitor for cGMP as well as cAMP (11, 21, 22), was without effect on cellular cGMP or cAMP levels or on secretion of resting lobules. No consistent enhancement in secretion was seen when theophylline at 10^{-5} and 10^{-4} M was added to lobules stimulated suboptimally with 10^{-6} M carbachol. Under these conditions, cellular cAMP and cGMP were both increased. Imidazole, claimed to be preferentially a cGMP phosphodiesterase inhibitor in micromolar concentrations (22), at 10^{-3}–10^{-7} M, had no effect on cGMP, cAMP, or secretion of either resting or suboptimally stimulated lobules.

Effect of exogenous cyclic nucleotides and cyclic nucleotide analogues

Cyclic GMP or cAMP, in a concentration range of 10^{-2}–10^{-8} M, had no discernible effect on secretion of resting or suboptimally stimulated lobules even in the presence of 10^{-4} M theophylline. However, the dibutyryl and 8-bromo substituted analogues of cGMP as well as of cAMP, in concentrations of 10^{-2} and 10^{-3} M, stimulated resting lobule secretion to the extent of 33 to >60% of the response to an optimal dose of carbachol (Fig. 7, upper panels). cAMP derivatives were somewhat more effective than cGMP derivatives, and the 8-bromo substituted analogues were more potent than the dibutyryl ones. This stimulation was not blocked by atropine at 10^{-4} M, but was abolished by 10^{-4} M CCCP.

Stimulation of resting lobules by 8-bromo cAMP at 10^{-3} M brought a phasic rise in endogenous cellular cGMP similar to that seen in suboptimal stimulation with carbachol (Fig. 7, lower panel; Fig. 3, upper panel left).

DISCUSSION

Previous studies on the role of cAMP in enzyme secretion by the exocrine pancreas (1–8) have been hindered by (*a*) methodological problems in the assay of cyclic nucleotides, (*b*) difficulties in distinguishing enzyme from fluid and electrolyte secretion, and (*c*) the use of nonphysiologically high concentrations of cAMP and its analogues, administered to the extracellular space either *in vivo* or *in vitro* (3, 5, 8, 23, 24). In the studies presented here, direct and specific measurement, by radioimmunoassay, of cellular cAMP and cGMP levels was used in a model system well defined for study of pancreatic enzyme secretion (15). Endogenous cGMP levels were found in close correlation with the secre-

tory response (*a*) during the time of onset of stimulation with cholinergic (carbachol) and peptide secretagogues (pancreozymin and caerulein) in concentrations approximating those operating *in vivo*, (*b*) during time of termination of carbachol stimulation, (*c*) over a concentration range, 10^{-8}–10^{4} M, of carbachol, and (*d*) in the use of specific (atropine) or nonspecific (CCCP) inhibitors of hormone-stimulated secretion. These findings contrast sharply with the nonspecific changes observed in cAMP levels. The coupling between cGMP levels and the secretory response in both guinea pig and rabbit pancreas lead us to conclude that cGMP is an intracellular mediator during stimulus secretion coupling in the acinar cell of the exocrine pancreas. Other workers (1, 8, 25) have recently defined a role for cAMP in the mediation of fluid and electrolyte secretion by the centro-acinar or duct cells. Taken together, the latter and our results would now explain most of the previously reported conflicting findings. The evidence derived from the application of very high concentrations (10^{-3}–10^{-2} M) of exogenous cyclic nucleotides and cyclic nucleotide analogues is, in our opinion, of little physiological relevance in the pancreatic system as well as in other systems. In this study, the application of 10^{-3} M 8-bromo cAMP produced a secretory response similar to suboptimal carbachol stimulation but also produced a rise in endogenous cGMP levels. Conversely, in other systems, application of nonphysiologically high concentrations of exogenous cGMP derivatives resulted in cAMP-like effects (23, 24). Similarly, evidence derived from studies with the phosphodiesterase inhibitors theophylline and imidazole, particularly when applied to cGMP systems, is open to serious questions due to the uncertain permeability of these agents and their potential multiple side effects.

Secretagogue action is thought to be initiated at the plasma membrane by secretagogue receptor interaction with membrane-bound cyclases. The additive effects seen with suboptimal carbachol and caerulein stimulation on cGMP levels and secretory response, as well as the specific inhibition seen with atropine of carbachol and not pancreozymin stimulation, suggest that separate receptors exist in the acinar cell membrane for acetylcholine and pancreozymin. The lack of additive effects of optimal carbachol and caerulein stimulation suggests that the two receptors interact with a common guanylate cyclase. Recent studies have localized cGMP, and by implication guanylate cyclase, in plasma membranes of cGMP-mediated cells using nondisruptive immunohistochemical methods (26). Cyclic GMP may lead to exocytosis through a series of steps, one of which may be the phosphorylation of specific membrane proteins by a cGMP-dependent protein kinase (27). The activation of such intermediate steps by cGMP, as well as rapid intracellular degradation of cGMP (23), would account for the differences seen in cellular levels of cGMP and rates of secretion over time. Similar differences in nucleotide levels and physiological response over time have been observed in other systems mediated by cAMP as well as cGMP (23). Changes in cellular calcium flux and membrane depolarization have been recognized as other steps in stimulus secretion coupling. The relationship between these events and cGMP formation in the pancreatic acinar cell remains to be elucidated.

We acknowledge the competent technical assistance by Eric Nestler, Geoffrey Ling, Michael Wright, Peony Lam, and Morris Milman. We thank Dr. G. E. Palade for stimulating discussions and helpful suggestions in the course of this work. This research was supported in part by Grant AM11431 from the National Institutes of Health, U.S. Public Health Service.

1. Case, R. M., Johnson, M., Scratcherd, T. & Sharett, H. S. A. (1972) *J. Physiol. (London)* **223**, 669–684.
2. Benz, L., Eckstein, B., Matthews, E. K. & Williams, J. A. (1972) *Br. J. Pharmac.* **46**, 66–77.
3. Kulka, R. G. & Sternlicht, E. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 1123–1128.
4. Knodell, R. G., Toskes, P. P., Reber, H. A. & Brooks, F. P. (1970) *Experientia* **26**, 515–517.
5. Ridderstap, A. S. & Bonting, S. L. (1969) *Pflugers Arch.* **313**, 62–70.
6. Heisler, S., Fast, D. & Tenenhouse, A. (1972) *Biochim. Biophys. Acta* **279**, 561–572.
7. Bauduin, H., Rochus, L., Vincent, D. & Dumont, J. E. (1971) *Biochim. Biophys. Acta* **252**, 171–183.
8. Case, R. M. & Scratcherd, T. (1972) *J. Physiol. (London)* **223**, 649–667.
9. Lee, Tee-Ping, Kuo, J. F. & Greengard, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3287–3291.
10. George, W. J., Polson, J. B., O'Toole, A. B. & Goldberg, N. D. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 398–403.
11. Goldberg, N. D., Haddox, M. K., Hartle, D. K. & Hadden, J. W. (1973) in *Proceedings of the Fifth International Congress on Pharmacology, San Francisco, California*, ed. Acheson, G. H. (S. Karger AG, Basel), Vol. 5, pp. 146–166.
12. Schultz, G., Hardman, J. G., Davis, J. W., Schultz, K. & Sutherland, E. W. (1972) *Fed. Proc.* **31**, 440.
13. Ignarro, L. J., Lint, T. F. & George, W. G. (1974) *J. Exp. Med.* **139**, 1395–1414.
14. Steiner, A. L., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113.
15. Scheele, G. A. & Palade, G. E. (1975) *J. Biol. Chem.* **250**, 2660–2670.
16. Haymovits, A., Scheele, G. A. & Nestler, E. (1975) *Fed. Proc.* **34**, 332.
17. Burton, K. (1956) *Biochem. J.* **62**, 315–322.
18. Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48–53.
19. Jamieson, J. D. & Palade, G. E. (1971) *J. Cell Biol.* **48**, 503–522.
20. Steiner, A. L., Pagliara, A. S., Chase, L. R. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1114–1120.
21. Goldberg, N. D., Larner, J., Sasko, H. & O'Toole, H. G. (1969) *Anal. Biochem.* **28**, 523–544.
22. Ishikawa, E., Ishikawa, S., Davis, W. J. & Sutherland, E. W. (1969) *J. Biol. Chem.* **244**, 6371–6378.
23. Goldberg, N. D., O'Dea, R. F. & Haddox, M. K. (1973) "Cyclic GMP," *Adv. Cyclic Nucleotide Res.* **3**, 156–213.
24. Berridge, M. J. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 2–98.
25. Smith, P. A. & Case, R. M. (1975) *Biochim. Biophys. Acta* **399**, 277–290.
26. Ong, S. H., Whitley, T. H., Stowe, N. W. & Steiner, A. L. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 2022–2026.
27. Casnellie, J. E. & Greengard, P. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1891–1895.