Hormone-induced Protein Phosphorylation. I. Relationship between Secretagogue Action and Endogenous Protein Phosphorylation in Intact Cells from the Exocrine Pancreas and Parotid

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ABSTRACT We undertook studies to determine whether secretagogue action on the exocrine pancreas and parotid is accompanied by phosphorylation of proteins in intact cells. For this purpose, rat pancreatic, and parotid lobules were preincubated with $^{32}P_i$ for 45 min at 37°C, washed, and then incubated at 37°C in the presence or absence of secretagogues that effect discharge through different second messengers. Among a variety of polypeptides exhibiting enhanced phosphorylation in pancreatic lobules upon a 30-s incubation in the presence of the secretagogues carbamylcholine, cholecystokinin octapeptide, or secretin, one species with an M_r of 29,000 was especially notable for three reasons: (*a*) its enhanced level of phosphorylation was dependent on the dose of secretagogue used and was still apparent after incubation for 30 min at 37°C; (*b*) an analogous phosphorylated polypeptide was observed in isoproterenol-stimulated parotid lobules; and (*c*) in both tissues its selective dephosphorylation was observed upon termination of stimulation by administration of atropine to carbamylcholine-stimulated pancreatic lobules and propranolol to isoproterenol-stimulated parotid lobules. These results suggest that the phosphorylation of one protein with an M_r of 29,000 is closely correlated both temporally and in a dose-dependent fashion with secretagogue action in both the exocrine pancreas and parotid.

The mechanism whereby secretagogues are able to elicit a characteristic biological response in their respective target cells is unclear at the moment. There is now increasing evidence that protein phosphorylation is involved in mediating the effects of a variety of the actions of hormones in many diverse enzymological and physiological processes (1). To gain further insight into the mechanism of secretagogue action in exocrine glands, we examined the relationship between protein phosphorylation and hormone action in the intact cell to determine whether this covalent modification mediates or modulates any of the biological actions of secretagogues.

The acinar cells of the exocrine pancreas and parotid offer good systems for studying secretagogue effects on endogenous protein phosphorylation since homogeneous cell preparations can be prepared, several different secretagogues exist which elicit discharge through different intracellular messengers, and the effects of these hormones on calcium and cyclic nucleotide levels during secretion are well characterized (2, 3, 4). Since cAMP, cGMP, and Ca^{2+} have been implicated in the secretion of exportable proteins from the exocrine pancreas, protein kinases are plausible targets for these putative secretagogue mediators and, in fact, both cAMP and cGMP-dependent protein kinases have been partially purified from homogenates of rat pancreas (5, 6).

We examined the relationship between endogenous protein phosphorylation and secretagogue action in situ in gland lobules under physiological conditions using the rat exocrine pancreas and parotid as model systems. A preliminary note on this research has been published (7).

MATERIALS AND METHODS

All chemicals used were of reagent grade and were obtained from the following sources: carbamylcholine chloride (carbachol), isoproterenol hydrochloride, atropine, propranolol, TRIS, HEPES, and dibutyryl cAMP were from Sigma Chemical Co. (St. Louis, MO); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 12-O-tetradecanoyl-phorbol-13-acetate from Aldrich Chemical Co. (Milwaukee, WI); and

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soybean trypsin inhibitor from Worthington Biochemical Co. (Freehold, NJ). 4,5-^{3}H-L-leucine (5 mCi/ml, 45 Ci/mmol) was obtained from Schwartz/Mann (Orangeburg, NY); carrier-free $^{32}P_i$ ($H_3{}^{32}PO_4$) (285 Ci/mg; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear (Boston, MA). Cholecystokinin octapeptide (CCK-8) and synthetic secretin were generous gifts from Dr. Miguel Ondetti (Squibb Institute for Medical Research, Princeton, NJ). A23187 was a generous gift from Dr. Robert L. Hamill of Eli Lilly Research Laboratories (Indianapolis, IN).

Sample Preparation

Sprague-Dawley female rats weighing 100–180 g and fed ab libitum were killed by a blow to the head, and the pancreas or parotid was excised within 2 min and immersed in oxygenated Krebs-Ringer HEPES medium (KRH) (104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 2.5 mM glucose, 25 mM HEPES, pH 7.4, 0.1 mg/ml soybean trypsin inhibitor) at 4°C. No amino acids were included in the medium due to their interference with the fluorescamine assay. Phosphate ions were excluded as they would compete with $^{32}P_i$ during prelabeling of the lobules.

Labeling of Intact Cells with $^{32}P_i$

Oxygenated KRH was injected into the loose connective tissue of the gland at 4°C to spread apart the lobules (8, 9), and each lobule was then excised under a dissecting microscope so that all of them were similar in size. Individual lobules (~80 μg of protein) were placed in separate test tubes in an ice-bath, and 45 μl of $^{32}P_i$ (5 mCi carrier free $^{32}P_i$) in oxygenated KRH was added to each. The samples were preincubated in uncapped tubes for 45 min at 37°C with shaking (150 oscillations/min) to incorporate the radioactive phosphate into cellular ATP pools, washed free of $^{32}P_i$ with oxygenated KRH at 4°C, and subsequently incubated in the presence or absence of secretagogues (in oxygenated KRH medium) for various lengths of time at 37°C in a final volume of 200 μl. It was found that prelabeling the lobules in a 100% O_2 phase increased incorporation of $^{32}P_i$ approximately tenfold. Since preincubation under O_2 produced the same protein phosphorylation pattern as observed with samples prelabeled under room air, this latter procedure was routinely used for convenience. The reactions were terminated by the addition of 50 μl of 10% SDS (2% final concentration), and all samples were boiled for 5 min to inactivate proteases at the end of the incubation period. The lobules were dispersed by sonication with a Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) equipped with a No. 422 Microtip and again boiled for 5 min to ensure complete solubilization. The amount of protein in each tube was determined by the fluorescamine assay (10), and to 125 μl of solubilized protein in 2% SDS was added 25 μl of a solution containing 25% sucrose, 75 mM EDTA, 750 mM TRIS, pH 7.5, pyronin Y, and 12% β-mercaptoethanol. Each tube was then adjusted to the same protein concentration and subjected to SDS PAGE on 9% resolving gels as follows. The resolving gel solution contained 426 mM TRIS, pH 8.95, 2.2 mM EDTA, 0.11% SDS, 9% acrylamide, 0.1% Temed, and 0.15% ammonium persulfate. The spacer gel solution contained 71 mM TRIS, pH 6.95, 2.2 mM EDTA, 0.11% SDS, 5% acrylamide, 0.1% Temed, and 0.15% ammonium persulfate. The electrophoresis buffer contained 475 mM glycine, 50 mM TRIS, 20 mM EDTA, and 0.1% SDS, the final pH of the solution being adjusted to 8.75 with NaOH. Electrophoresis was performed at 130 V for ~4 h at room temperature. The gels were stained for 1 h with 0.06% Coomassie Blue in 50% methanol plus 10% acetic acid and destained overnight in 10% isopropanol plus 10% acetic acid. After drying, they were placed on Kodak XRP-1 x-ray film with DuPont Lightening Plus intensifying screens (DuPont Instruments, Wilmington, DE) for 12 to 24 h to detect proteins that incorporated $^{32}P_i$.

Secretory Protein Discharge Assay

To analyze secretory protein discharge, pancreatic lobules were pulse-labeled for 5 min with [^{3}H]leucine at 37°C in oxygenated KRH medium containing 0.5 mM potassium phosphate and chased for 45 min at 37°C in KRH medium containing 0.5 mM potassium phosphate plus 7 mM unlabeled leucine to allow radio-labeled secretory proteins to reach zymogen granules (12). The lobules were washed and incubated for various times at 37°C in the presence or absence of secretagogues, and the TCA-precipitable radioactivity in the lobules and medium was quantitated (12). Discharge was expressed as the percentage of the total radioactivity in proteins (tissue and medium) which appeared in the medium. Secretion was measured identically for parotid lobules except that the chase period was extended to 150 min since the maturation time for secretory granules in the parotid is longer than in the pancreas (9).

Other Methods

Radioactive nucleotides from SDS-treated and sonicated lobules were analyzed by thin-layer chromatography on PEI-cellulose (J. T. Baker Chemical Co., Phillipsburg, NJ) sheets using 1.6 M LiCl as the resolving solvent (13). After drying, the $^{32}P_i$-labeled nucleotides were localized by autoradiography and the corresponding spots scraped off the plates and quantified by liquid scintillation spectrometry.

RESULTS

Endogenous Protein Phosphorylation and Secretagogue-induced Discharge in Pancreatic Lobules

When pancreatic lobules were incubated with KRH alone or in the presence of 0.1 nM CCK-8, 10 μM carbachol, or 1 μM secretin for 30 s at 37°C, a marked stimulation of the endogenous phosphorylation of several proteins with apparent molecular weights of 62,000, 55,000, 52,000, 49,000, 34,000, and 29,000 was observed (Fig. 1). However, after a 30-min incubation at 37°C, the endogenous phosphorylation of principally one protein with an apparent mass of 29,000 daltons was maintained at levels comparable to that observed following 30 s of secretagogue action. The enhanced phosphorylation of this protein was seen irrespective of the secretagogue used, suggesting that there may be a relationship between the ability of these agents to effect discharge and the ability to stimulate protein phosphorylation. At this point, we decided to concentrate on the 29,000-dalton polypeptide because of the prominent hormone effects on the phosphorylation of this protein at 30 min and the stability of the incorporated $^{32}P_i$ over time. This does not infer, however, that the other phosphoproteins described in Fig. 1 are not of physiological importance, rather, the 29,000-dalton phosphoprotein shows unique properties that allow it to be singled out on temporal considerations.

To define the relationship between the phosphorylation of the 29,000-dalton polypeptide and hormone action, the effects of various concentrations of each secretagogue on protein discharge and endogenous protein phosphorylation were analyzed, protein discharge being used only as an assay for secretagogue response and not for exocytosis *per se*. The data show a close correlation between the dose-response curves for the

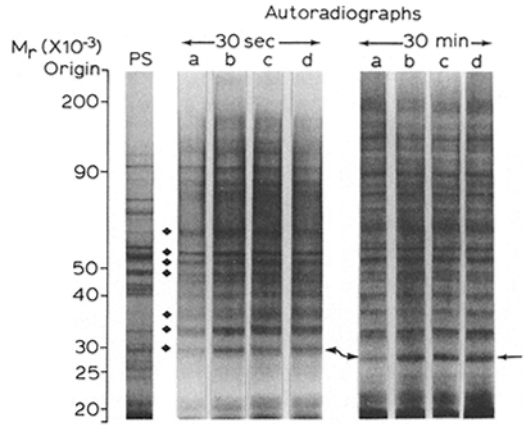

FIGURE 1 Protein pattern (*PS*) and autoradiographs of rat pancreatic lobules incubated for 30 s or 30 min at 37°C with KRH medium alone (*a*), 0.1 nM CCK-OP (*b*), 10 μM carbachol (*c*), or 1 μM secretin (*d*). Apparent M_r values were determined by comparison to known standards run on the same gel-cytochrome C (12,500), chymotrypsinogen A (25,000), carbonic anhydrase (29,000), ovalbumin (45,000), BSA (68,000), and myosin (220,000). The 30-s and 30-min samples were electrophoresed on separate gels, the 29,000-dalton phosphoprotein being indicated by arrows. The diamonds indicate the proteins whose phosphorylation was consistently observed to be enhanced by secretagogues at the 30-s time point.

stimulation of phosphorylation of the 29,000-dalton protein and secretion (Fig. 2). The results indicate that the the dose-response curves for secretion and for phosphorylation of the 29,000-dalton protein exhibit half-maximal effects induced by carbachol, CCK-8, and secretin at ~0.1 μM, 0.1 nM, and 0.1 μM, respectively. After a 1-h incubation with 10 μM carbachol at 37°C, the hormone-response phosphoproteins including the 29,000-dalton species were not found in the incubation medium and did not comigrate with proteins discharged from lobules. Thus, these phosphoproteins are not secretory proteins discharged in response to hormone application. A correlation between induction of secretion and protein phosphorylation was also seen for the other proteins shown in Fig. 1 whose phosphorylation was enhanced at the 30-s time point (data not shown).

Since there was a good dose-response correlation between secretagogue-induced discharge and protein phosphorylation, the temporal relationship between these two processes was examined in detail for carbachol. Significant secretion over basal values (KRH alone) was also seen within 30 s and proceeded continuously during the 1-h assay (Fig. 3*A*). In parallel, the onset of endogenous phosphorylation of the 29,000-dalton protein was rapid, a maximal response being reached within 30 s and maintained throughout the course of carbachol application (Fig. 3 *C*). Only the 29,000-dalton phosphoprotein was consistently observed to be stimulated during the entire time course of secretagogue application both for carbachol and for CCK-8 (data not shown).

Enhanced phosphorylation was not attributable to an increase in radioactive ATP levels within the cell (Table I). In addition, the phosphate incorporated into this protein appears to be metabolically stable for at least 60 min, (based on two observations). First, the levels of the radioactive nucleotide decreased over time in a secretagogue-independent fashion with no effect on $^{32}P_i$ incorporated into the 29,000-dalton species. Second, addition of nonradioactive phosphate to the media after the initial $^{32}P_i$ labeling of the lobules had no effect on secretagogue-enhanced labeling of this protein (data not shown). The other phosphoproteins described in Fig. 1 exhibited maximal enhancement of phosphorylation within 10 to 30 s after addition of secretagogues but did not show sustained secretagogue responsiveness over time. Thus, the 29,000-dalton polypeptide appears to be the principal protein whose phosphorylation correlates temporally and also in a dose-dependent fashion with secretagogue-induced discharge.

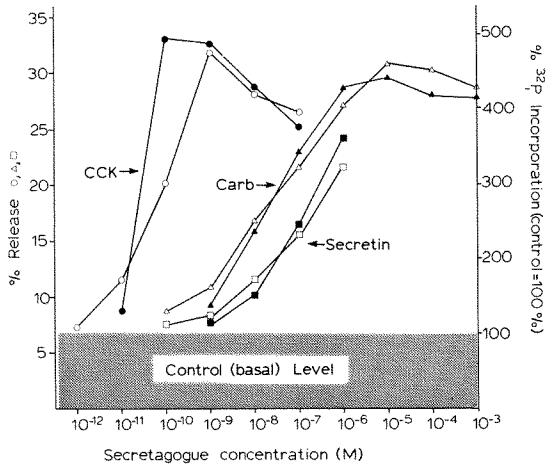

FIGURE 2 Dose-response curves for endogenous protein phosphorylation and ^{3}H-leucine release in rat pancreatic lobules. For protein phosphorylation studies, lobules prelabeled with $^{32}P_i$ were incubated for 30 s at 37°C with various concentrations of secretagogues and analyzed by SDS PAGE. Autoradiographs were densitometrically scanned and results are expressed as $^{32}P_i$ incorporation into the 29,000-dalton protein. Values are expressed as percent of control where control equals 100% (KRH medium alone) and represent the average of six experiments each analyzed in duplicate. For secretion studies, two lobules were placed in each tube and percent release of acid-precipitable radioactivity was measured after a 30-min incubation at 37°C with various concentrations of secretagogues as described in Materials and Methods. Each value is the average of three separate experiments each analyzed in duplicate. (●) CCK-induced phosphorylation. (▲) Carbachol-induced phosphorylation. (■) Secretin-induced phosphorylation. (○) CCK-induced secretion. (△) Carbachol-induced secretion, (□) Secretin-induced secretion.

Lobules, consisting of >90% acinar cells, still contain duct cells, centroacinar cells, capillaries, and endocrine cells. To rule out the possibility that the proteins phosphorylated were not contributed by this population of nonacinar cells, pancreatic acini were prepared by collagenase and mechanical dissociation (14), and phosphorylation studies were performed as described for lobules. This cell preparation, while containing ~2% centroacinar cells, is devoid of endocrine cells, small blood vessels, and ductules. All proteins that were phosphorylated in lobules were also seen in the acinar preparation, with some additional higher molecular weight phosphoproteins observed in the latter (data not shown). Thus, the phosphoproteins described in the lobule preparation including the 29,000-dalton species are indeed indigenous to the acinar cell.

Endogenous Protein Phosphorylation and Secretory Protein Discharge in Parotid Lobules

To investigate whether proteins phosphorylated in response to secretagogues in the pancreatic acinar cell are present in other secretory cells, the parotid, an exocrine tissue consisting of 91% acinar cells (volume per lobule [9]), was analyzed. Protein phosphorylation studies were performed exactly as for pancreatic lobules except that the secretagogue used was 1 μM isoproterenol (a dose which elicits maximal discharge). The protein and phosphorylation patterns from parotid and pancreatic lobules are compared in Fig. 4. 30 s after hormone addition, the endogenous phosphorylation of several proteins in the parotid was enhanced. In contrast, after a 30-min incubation with 1 μM isoproterenol, the phosphorylation of only one protein with an apparent M_r of 29,000 was markedly stimulated while in the basal condition (KRH medium alone) it was dephosphorylated. The retention of enhanced labeling of this species despite the presence of 1 mM unlabeled phosphate in the medium after $^{32}P_i$ labeling of lobules and the declining levels of labeled ATP observed during incubation (Table I) suggest that the incorporated phosphate is relatively stable over the 60-min time course examined. This protein comigrated with the 29,000-dalton polypeptide described in the pancreatic acinar cell, suggesting that the same protein in two different exocrine systems is affected by secretagogue application.

Quantification of the effect of isoproterenol and of dibutyryl cAMP, another parotid secretagogue, on secretory protein discharge and phosphorylation is shown in Table II. 1 μM isoproterenol evoked a 15-fold increase in discharge over basal values while dibutyryl cAMP was ~50% as effective as isoproterenol. Similar results were observed for the endogenous phosphorylation of the 29,000-dalton protein where dibutyryl cAMP was approximately one-half as effective as iosproterenol. Thus, the

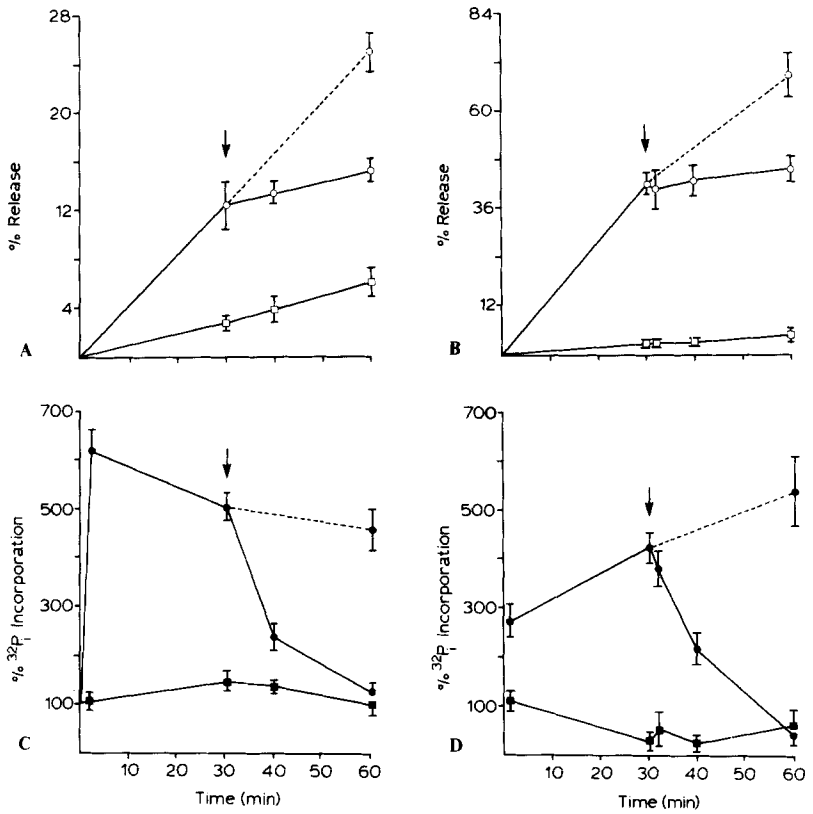

FIGURE 3 Atropine reversal of carbachol-induced (*A*) discharge and (*C*) endogenous phosphorylation of the 29,000-dalton protein in pancreatic lobules and propranolol reversal of isoproterenol-induced (*B*) discharge and (*D*) endogenous phosphorylation of the 29,000-dalton protein in parotid lobules. $^{32}P_i$-labeled lobules were incubated in the presence or absence of 10 μM carbachol or 1 μM isoproterenol in the pancreas and parotid, respectively, for 30 min at 37°C. At this point either KRH (dashed line), 1 mM atropine or 100 μM propranolol was added at 30 min (indicated by the arrow) to the respective tissues and the reaction allowed to continue for an additional 30 min at 37°C. After SDS PAGE, autoradiographs were either densitometrically scanned or polypeptides were cut out from the gel, disolved in 30% H_2O_2 at 110°C for 12 h, put into Aquasol scintillation fluid and counted for 10 min in a Beckman LS-150 scintillation counter. Both methods gave similar results. Each data point represents the mean ± SD from three separate experiments, each analyzed in duplicate. 100% represents on the average 85 cpm/10 μg total cellular protein in the pancreas and 112 cpm/10 μg total cellular protein in the parotid, where 100% represents percent $^{32}P_i$ incorporated into the 29,000-dalton protein at 0 min for the pancreas and at 30 s for the parotid. For secretion studies, three lobules were placed in each flask on a nylon mesh in a 2-ml reaction volume (18) and incubated at 37°C as described for the phosphorylation studies. At the specified times, 0.5 ml of incubation medium was removed and immediately replaced by the same volume of fresh medium. Values are expressed as percent cumulative release and each data point represents the average of three separate experiments each analyzed in duplicate. Carbachol-induced secretion (○), basal secretion (□), carbachol-induced phosphorylation (●), basal phosphorylation (■). Isoproterenol-induced secretion (○), basal secretion (□), isoproterenol induced phosphorylation (●), basal phosphorylation (■).

stimulation of endogenous phosphorylation of the 29,000-dalton polypeptide in the parotid occurs concomitant with secretion, analogous to the situation in the pancreatic acinar cell.

Reversal of Secretagogue-induced Discharge and Protein Phosphorylation

Discharge from pancreatic lobules was initiated with 10 μM carbachol and allowed to continue for 30 min (Fig. 3*A*). When a 100-fold excess of the cholinergic antagonist atropine was subsequently added, no further change in the cumulative release of TCA-precipitable ^3H-labeled proteins was seen 10 min later, indicating that secretagogue effects had been terminated. Investigation of the endogenous phosphorylation of the 29,000-dalton protein under identical conditions demonstrated that this protein was dephosphorylated when carbachol-induced secretion was reversed by atropine, the lag time being similar for both events (Fig. 3*C*). The 29,000-dalton protein appeared to be the principal protein dephosphorylated under these conditions.

These studies were extended to the parotid. Isoproterenol-induced secretion from parotid lobules was allowed to continue for 30 min and then 100 μM propranolol, a specific β-adrenergic antagonist, was added (Fig. 3*B*). Within 10 min after propranolol addition, discharge was terminated as evidenced by no further increase in the release of TCA-precipitable ^3H-labeled proteins. Under these same conditions, only the 29,000-dalton protein was significantly dephosphorylated within 10 min after the addition of propranolol (Fig. 3*D*). These data reinforce the close temporal relationship between secretagogue action and the endogenous phosphorylation of the 29,000-dalton protein as well as termination of secretagogue effects and subsequent dephosphorylation of this same protein.

DISCUSSION

In this study, we describe the endogenous phosphorylation of a 29,000-dalton protein in pancreatic and parotid lobules exposed to secretagogues. Our finding represents an effect in two exocrine tissues that reflects the interaction of a secretory stimulus with the cell. Since this specific covalent modification constitutes a correlation in time and with secretagogue dose as reflected by secretory protein discharge, we designed experiments to test whether these two events are functionally related. It is important to emphasize that, in addition to eliciting exocytosis, secretagogues may affect other cellular processes such as receptor modulation and function, transport across membranes, carbohydrate metabolism, cytoskeletal function, and the modulation of mRNA-directed synthesis of secretory proteins (1). Thus, the demonstration of a relationship between secretagogue action and protein phosphorylation does not allow one to ascribe this latter process to a specific cellular response without further data.

In the exocrine pancreas, there appears to be at least two different pathways by which secretagogues can effect protein discharge as reviewed in (2, 3). Secretin, vasoactive intestinal peptide, and cholera toxin appear to activate adenylate cyclase and cause increased levels of cAMP with no effect on Ca^{2+} fluxes. In contrast, carbachol, CCK-8, and bombesin induce the release of intracellular Ca^{2+}, as well as cause increased levels of cGMP, although the latter effect has been shown not

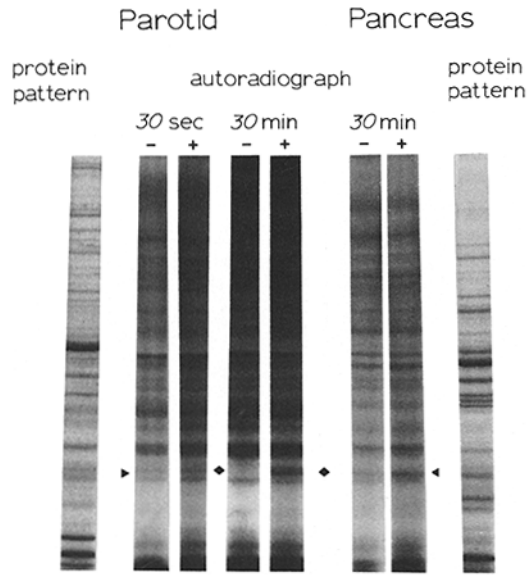

FIGURE 4 Protein patterns and autoradiographs of parotid and pancreatic lobules prelabeled with $^{32}P_i$. For the parotid, + represents 1 μM isoproterenol. For the pancreas, + represents 0.1 nM CCK-8. In both cases, − is KRH medium alone. The position of the 29,000-dalton phosphoprotein is indicated by the arrowheads.

TABLE I

Radioactive ATP Levels in $^{32}P_i$-labeled Lobules as a Function of Time and Secretagogue Application

	[γ-^{32}P]ATP levels			
	0 min	1 min	30 min	60 min
Pancreas				
KRH	1,463	1,391	1,186	1,047
0.5 nM CCK-8	1,447	1,382	1,209	1,038
Parotid				
KRH	1,504	1,491	1,374	1,148
1 μM isoproterenol	1,522	1,513	1,362	1,246

Lobules were prelabeled with $^{32}P_i$ for 45 min at 37°C, incubated in the absence or presence of secretagogues for either 0, 1, 30, or 60 min at 37°C, the reactions terminated with 2% SDS, and [γ-^{32}P]ATP measured as described in Materials and Methods. Values are expressed as cpm from one experiment and are qualitatively similar to results obtained in two other experiments. Samples assayed contained equal protein concentrations.

to correlate with secretory protein discharge from pancreatic acinar cells (15). Analogous to the proposed mechanism for secretin and vasoactive intestinal peptide-stimulated secretion in the pancreas, application of isoproterenol to acinar cells from the parotid results in activation of adenylate cyclase followed by an increase in cAMP production (4). In both systems, the endogenous phosphorylation of the same 29,000-dalton protein is enhanced irrespective of the secretagogue used. This implies that the 29,000-dalton protein may be phosphorylated by both Ca^{2+} as well as cAMP dependent protein kinases within the cell in response to secretagogue application. In addition, the occurrence of this phosphoprotein in the parotid as well as the exocrine pancreas suggests that the phosphorylation of the 29,000-dalton protein may be common to several exocrine tissues.

Recent work by Gunther and Jamieson (15) has shown that a 2-min incubation of pancreatic acini with 100 μM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine causes a 100-fold increase in cGMP levels without inducing discharge; under the same conditions no enhancement in protein phosphorylation was seen (data not shown). Furthermore, 100 nM 12-O-tetradecanoylphorbol-13-acetate causes secretion without a concomitant rise in cGMP, while the phosphorylation of the 29,000-dalton protein exhibited maximal stimulation (data not shown). Thus, cGMP does not appear to be directly involved in secretagogue action or protein phosphorylation in this system.

TABLE II

Effects of Isoproterenol and Dibutyryl cAMP on Protein Discharge and Endogenous Protein Phosphorylation in Parotid Lobules

	% release	% $^{32}P_i$ incorporation into 29,000-dalton protein
KRH	2.1 ± 0.8	100
1 μM isoproterenol	31.9 ± 3.4	392 ± 38
1 mM dibutyryl cAMP	17.8 ± 2.1	189 ± 21

Release of secretory proteins was measured after a 30-min incubation at 37°C as described in Materials and Methods. Values represent the mean ± SD from three experiments each analyzed in duplicate. $^{32}P_i$ incorporation was determined as in Fig. 3. 100% represents the $^{32}P_i$ incorporated into the 29,000-dalton protein after a 30-min incubation at 37°C in KRH medium and is on average equal to ~390 cpm/10 μg total cellular protein. All other values are expressed as the mean ± SD and are representative of five independent experiments.

Further support for the involvement of Ca^{2+} in secretagogue response and protein phosphorylation in the exocrine pancreas comes from data utilizing the ionophore A23187 which, in the presence of extracellular Ca^{2+}, raises cytosolic Ca^{2+} levels and stimulates amylase release from acini (16, 17). A23187 (0.5 μM) elicits maximal discharge of TCA-precipitable ^{3}H-labeled proteins from lobules and simultaneously stimulates the endogenous phosphorylation of this protein (data not shown). Since the ionophore bypasses the proximal steps in secretagogue action, i.e., binding of the hormone to specific receptors on the cell surface, phosphorylation of the 29,000-dalton polypeptide may represent a more distal event in secretagogue action.

Recently, Jahn et al. (11), using $^{32}P_i$-labeled rat parotid slices, have demonstrated that the phosphorylation of three proteins with apparent molecular weights of 35,100, 25,700, and 20,700 was stimulated to the same extent by either isoproterenol or dibutyryl cAMP after a 30-min incubation at 37°C. With mouse parotid gland slices, only a 35,100-dalton protein exhibited an increase in labeling after incubation with either isoproterenol or carbachol, prompting the authors to postulate that the phosphorylation of this specific protein may play an important role in exocytosis (19). This protein was localized to a smooth membrane fraction and exhibited a basic pI. As will be shown in the following paper, the 29,000-dalton phosphoprotein described in our study is ribosomal protein S6 and may be related to the 35,100-dalton protein described by Jahn et al. (11). However, the ribosomal nature of this protein precludes a direct involvement of this specific covalent modification with the secretory aspect of hormone action.

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