Subcellular Location of Stimulus-affected Endogenous Phosphoproteins in the Rat Parotid Gland

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ABSTRACT Rat parotid minces were labeled with [32P]Pi, stimulated with isoproterenol, homogenized in sucrose, and fractionated on continuous sucrose density gradients. We analyzed the resulting fractions by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiograms were made from the gels. Comparison of fractions from control and isoproterenol-stimulated minces revealed seven phosphoproteins that were affected by isoproterenol. The subcellular location of these proteins was determined by comparing their distribution in the sucrose gradients with that of a number of enzymes that are characteristic of specific organelles. Isoproterenol decreased the phosphorylation of two cytoplasmic proteins (Mr 16,000 and 18,000) and increased the phosphorylation of a third (Mr 14,000). The phosphorylation of two endoplasmic reticulum proteins was increased by isoproterenol (Mr 20,500 and 22,500), as was an Mr 31,000 protein which was probably the S6 ribosomal protein. The phosphorylation of a secretory granule protein (Mr 24,000) was decreased by isoproterenol. We then developed a purification scheme for parotid secretory granules. By using this method, we demonstrated that the phosphorylation of the Mr 24,000 was also decreased by carbamylcholine. Granules purified by this method also contained a small number of other phosphoproteins whose phosphorylation was increased only by isoproterenol. Secretory granule-associated stimulus-affected phosphoproteins were found in the particulate fraction when the granules were hypotonically lysed, and were not extracted from the particulate fraction by washing with 0.6 M KCl.

β-Adrenergic stimulation is the major inducer of exocytosis in the rat parotid gland, although choliner gic and α-adrenergic stimulation also produce limited amylase release (1). β-Adrenergic agonists apparently exert their effects by activating parotid adenylate cyclase (2), thereby increasing cyclic AMP levels (3), and activating cyclic AMP-dependent protein kinase (4, 5). It has been demonstrated, by incubating parotid tissue with [32P]Pi, stimulating with neurotransmitters, and analyzing radiolabeled phosphoproteins by electrophoresis and autoradiography, that neurotransmitters affect the phosphorylation of several endogenous parotid phosphoproteins (6–10). However, because neurotransmitters affect several processes in salivary glands in addition to exocytosis, e.g., ion fluxes (11), amino acid transport (12), protein synthesis (13), cellular proliferation (14), and energy metabolism (15), it is difficult to ascribe these stimulus-affected phosphoproteins to any particular glandular function. A knowledge of the subcellular localization of these stimulus-affected phosphoproteins may provide clues to which ones are potential intermediates in stimulus-secretion coupling, and which others are probably involved in other functions. The only parotid stimulus-affected phosphoprotein whose subcellular location is presently known has been shown to be the ribosomal S6 phosphoprotein (16, 17), an unlikely candidate for a role in stimulus-secretion coupling.

MATERIALS AND METHODS

Materials

All chemicals used were of reagent grade. Sialic acid-free, galactose-free fetuin was donated by Dr. J. P. Durham, Departments of Surgery and Biochemistry, West Virginia University. [γ-32P]ATP was synthesized by the method of Johnson and Wäbisch (18) using enzymes from Boehringer Mannheim Bio-
chemicals, Indianapolis, IN, and purified by the method of Palmer and Avruch (19). [γ-32P]ATP and [32P]PI were from New England Nuclear, Boston, MA. [14C]Cyclic AMP and UDP-[6-3H]galactose were from Amersham Corp., Arlington Heights, IL. Electrophoresis reagents, molecular weight markers, and diithiothreitol were from Bio-Rad Laboratories, Rockville Center, NY. Glutaraldehyde, propylene oxide, and uranyl acetate were from Polysciences, Inc., Warrington, PA. Epox 812 was from E. F. Fullam, Inc., Schenectady, NY. Other biochemicals and enzymes were from Sigma Chemical Co., St. Louis, MO.

Methods

**Tissue Preparation and Homogenization:** Parotid minces were prepared as previously described (20), except that KH2PO4 was omitted from the incubation media. Homogenates were prepared using four strokes in a motor-driven Teflon-glass homogenizer, using 1 ml of ice-cold homogenizing medium (0.3 M sucrose, 10 mM 4-(2 hydroxyethyl)-1-piperazine-ethanesulfonic acid-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM ethylene-glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)) for each 0.1 g of tissue (wet wt). This method has been reported to inhibit posthomogenization protein dephosphorylation with rat parotid (6). Connective tissue and unbroken cells were removed by centrifugation at 150,000 g (max) for 10 min at 4°C (IEC Centra-7R, International Equipment Co., Needham Heights, MA).

**Sucrose Density Gradients:** The supernatant from the preceding step was layered onto a 13.2-ml 0.3-2.0 M continuous sucrose density gradient composed of 2 ml of 1.7 M sucrose over 3.5 ml of 2.0 M sucrose made in a 17 × 111-mm polycarbonate centrifuge tube. Where indicated, the EDTA and EGTA was replaced with 1 mM MgCl2 and 1 mM CaCl2 in both the homogenizing medium and the gradient. The gradient was centrifuged at 4°C in a Beckman SW-28 rotor (Beckman Instruments, Inc., Fullerton, CA). The integrated areas of the isoproterenol-affected phosphoproteins in each fraction were corrected for slight differences in the sucrose density of the corresponding fractions from the "control" and "stimulated" gradients via a normalizing factor, calculated from the difference in the areas of a major phosphoprotein not affected by stimulation. For those proteins whose phosphorylation was increased by isoproterenol, the area of the protein in each control fraction was subtracted from its area in the corresponding stimulated fraction. The calculation was reversed for those proteins whose degree of phosphorylation was decreased by stimulation. Because most of the isoproterenol-affected phosphoproteins appeared in more than one gel, and because each gel was exposed for a different length of time, the difference calculated above was divided by the exposure time in days.

**Markers:** Monoamine oxidase was assayed by the method of Schnaitman et al. (23), except that 0.1% Triton X-100 was included in the assay and activity was determined at ambient temperature using (80 µl of each fraction) Galactosyltransferase was assayed by a modification of the method of Baxter and Durham (24). The assay contained 50 mM morpholinopropanesulfonic acid-KOH, pH 6.5, 15 mM MgCl2, 1 mM diithiothreitol, 200 µg of sialic acid-free, galactose-free fetuin, 50 µM UDP-D-[6-3H]galactose (2 µCi/µmol), 0.75% Triton X-100, and sample in a final volume of 60 µl. Samples consisted of 10 µl of each fraction precipitated by incubation for 60 min on ice with 10 µl of 1.5% Triton X-100. Incubation was at 30°C for 30 min and was terminated by spotting 50 µl of the reaction mix onto a 2.3-cm Whatman 3-mm disk (Whatman Chemical Separation Inc., Clifton, NJ) and immersing in ice-cold 10% trichloroacetic acid. After unincorporated radioactivity was washed off the disks, the retained radioactivity was solubilized with H2SO4 (24) and counted in a 

Sucinate dehydrogenase was assayed using 50 µl of each fraction by the method of Bachmann et al. (25), except that the reaction was performed at ambient temperature. Adenylate kinase was assayed with the method of Schnaitman and Greenwald (26) except that Lubrol activation was omitted and the assay was performed at ambient temperature. 50-µl aliquots of each fraction were used. β-glucuronidase was assayed with the method of Brittinger et al. (27), using 150 µl of each fraction. Fumarase was assayed by the method of Kasarke and Hill (28), except that 0.1% Triton X-100 was included in the assay. 25 µl of each fraction was used. NADPH cytochrome c reductase was assayed using 50-µl aliquots with the method of Omura et al. (29), except that the cytochrome c concentration was increased to 0.05 mM. 0.5 mM KCN was included in the assay, and the incubation was at ambient temperature. γ-Glutamyl transferase was assayed using γ-glutamyl-p-nitroaniline as described by Tate and Meister (30), using 50 µl of each fraction. RNA was assayed by the method of Munro and Fleck (31), using the entire volume of each fraction. Alkaline phosphodiesterase was determined in 50-µl aliquots of each fraction by the method of Ives et al. (32).

Glucose-6-phosphatase was assayed according to the method of Durham et al. (33). 50-µl aliquots of each fraction were assayed in a final volume of 100 µl. One aliquot was boiled before assay to serve as a blank. The incubation was for 30 min at 37°C and was terminated by the addition of 200 µl ice-cold 20% trichloroacetic acid. The samples were clarified by centrifugation and P, was determined in 200 µl of the supernatant by the method of Chen et al. (34). Adenylate cyclase was assayed by the method of Salomon et al. (35), using 50 µl of each fraction. Lactate dehydrogenase was assayed by the method of Stolzenbach (36), using 20 µl of each fraction. Alkaline phosphatase was...
assayed according to Ray (37) using 50 μl of each fraction. For the assay of amylas-
ity, 25 μl of each fraction was diluted to 500 μl with 1 mg/ml BSA in 6.7
mM NaCl and 20 mM phosphate buffer, pH 6.9. 25 μl of the diluted fractions
were then assayed by the method of Bernfeld (938).

Na/K ATPase was assayed by measuring the release of \[^{32}P\]Pi from \[^{32}P\]ATP. The assay contained 0.1 M Tris-HCl, pH 7.4, 60 mM NaCl, 5 mM KCl,
0.1 mM EDTA, 2 mM MgSO4, and 2 mM \[^{32}P\]ATP (0.25 μCi/ml) in a final
volume of 100 μl. 25 μl of each fraction was assayed in the absence and
presence of 1 mM ouabain; the difference was taken as the activity of Na/K
ATPase. The incubation was for 20 min at 37°C, and was terminated by the
addition of 1 ml ice-cold 10 mg/ml charcoal in 50 mM KH2PO4. The charcoal
was removed by centrifugation and 750 μl of the supernatant counted in 3.5
ml of ACS.

Roteone-sensitive NADH cytochrome c reductase was assayed by a mod-
fication of the method of Sottocasa et al. (39). The assay contained 50 mM
potassium phosphate, pH 7.5, 0.5 mM KCN, 0.05 mM rotenone, and 50 μl of fraction in a final volume of 1 ml.
The absorbance at 550 nm was monitored at ambient temperature.

5'-Nucleotidase was assayed by a modification of the method of Beaufay et
al. (40). The assay contained 50 mM HEPES-KOH, pH 8.2, 10 mM MgCl2, 5
mM AMP and 50 μl of sample in a final volume of 200 μl. Each fraction was
assayed in duplicate, with one aliquot boiled before assay to serve as a blank. The incubation was for 30 min at 37°C, and was terminated by the addition of
0.1 ml ice-cold 40% trichloroacetic acid. The samples were clarified by cen-
trifugation and the Pi content of 200 μl of the supernatant was determined by the
assay of Chen et al. (41) except that the assay volume was reduced to 200 μl. Duplicate 40-μl aliquots of each fraction were assayed, with one aliquot boiled before assay to serve as a blank. Liberated Pi was determined by the method of Chen et al. (34).

Thiamine pyrophosphatase was assayed by the method of Moldolesi et al.
(41) except that the assay volume was reduced to 200 μl. Duplicate 40-μl aliquots of each fraction were assayed, with one aliquot boiled before assay to serve as a blank. Liberated P; was determined by the method of Chen et al. (34).

Electron microscopy: Two glands were minced, homogenized, and
fractionated in a sucrose density gradient as described above. Frac-
tions containing 0.8-1.0 M sucrose were pooled, as were fractions containing 1.1-1.3
M, 1.4-1.55 M, and 1.75-1.85 M sucrose. The four pooled samples and purified
secretory granules were fixed by the addition of purified 70% glutaraldehyde,
with agitation, to a final concentration of 3%. After 1 h at room temperature,
they were diluted in half with 0.1 M HEPES, pH 7.2, and pelleted in conical
centrifuge tubes. The samples were then rinsed three times for 5 min each in
0.1 M HEPES, pH 7.2, and then postfixed with 1% OsO4 in 0.1 M HEPES,
pH 7.2, for 1 h at room temperature. After three buffer rinses, samples were
dehydrated through graded ethanol solutions, rinsed with propylene oxide, and
eMBEDDED in Epon 812. Thin sections were cut using diamond knives on a
Dupont-Sorvall MT-5000 ultramicrotome (Newton, CT), stained with 3.0%
(wt/vol) uranyl acetate and Reynold’s lead citrate (42). Microscopy was per-
formed with a JEOl 100 CX TEMSCAN (Peabody, MA) operated at 80 kV.

RESULTS

Sucrose gradient fractionation of control and isoproterenol-
stimulated \[^{32}P\]-labeled parotid minces revealed seven phos-
phoproteins that were affected by isoproterenol (Fig. 1). The approximate molecular weights of these proteins were 14,000,
16,000, 18,000, 20,500, 22,500, 24,000, and 31,000. Iso-
proterenol decreased the phosphorylation of the proteins with approximate molecular weights of 16,000, 18,000, and
24,000, whereas the phosphorylation of those remaining was
increased upon stimulation (Fig. 1).

The Mr. 16,000 and 18,000 phosphoproteins were found at the top of the gradients, with the largest amounts found in fractions containing approximately 0.3-0.4 M sucrose (Fig.
2, a and b). The Mr. 14,000 phosphoprotein appeared weakly labeled by comparison and penetrated slightly deeper into the
gradient; maximum amounts were found at ~0.4-0.5 M sucrose (Fig. 2c). The Mr. 31,000 phosphoprotein was highly
labeled and widely distributed, with a peak at 0.8-0.9 M sucrose (Fig. 2d). The Mr. 20,500 and 22,500 phosphoproteins
both peaked at ~1.1-1.2 M sucrose (Fig. 2, e and f) whereas the relatively weakly labeled Mr. 24,000 protein was found
near the bottom of the gradient at a sucrose concentration of
~1.7-1.8 M (Fig. 2g).

Unlabeled parotid minces were homogenized in the same
manner as the labeled minces and fractionated. The resulting fractions were assayed for several enzymes commonly used
as markers for various subcellular organelles. Lactate dehydro-
genase, a cytoplasmic marker (43), appeared at the top of the
gradient, peaking at 0.3-0.4 M sucrose (Fig. 3a). The plasma
membrane markers adenylyl cyclase (43), 5'-nucleotidase
(44), Na/K ATPase (43), \(^{3}2\)-glutamyl transpeptidase (45),
alkaline phosphodiesterase (43), and alkaline phosphatase (43)
all showed a similar distribution, with maximal activity found
at 0.8-0.9 M sucrose (Fig. 3, b-g). RNA also showed a major
peak at this sucrose concentration, which probably represents
ribosomes, and a smaller peak at 0.3-0.4 M sucrose, which
may be due to transfer RNA and/or ribosomal RNA degraded
by endogenous RNase (46) (Fig. 3h). \(^{3}2\)-Glucuronidase, a
lysosomal marker (47), exhibited a bimodal distribution with
peaks at sucrose concentrations of 0.5 and 1.4-1.5 M sucrose
(Fig. 3i). The heavier peak probably represents intact lys-
osomes whereas the higher peak may result from \(^{3}2\)-glucuronidase
released from lysosomes ruptured during tissue homog-
FIGURE 2 Distribution of isoproterenol-affected rat parotid endogenous phosphoproteins in sucrose density gradients in the presence of EDTA and EGTA. Conditions were as described in Materials and Methods. Results shown are from a single representative experiment.
FIGURE 3 Distribution of subcellular markers in sucrose density gradients in the presence of EDTA and EGTA. Conditions were as described in Materials and Methods. Results shown are compiled from several different experiments. Each marker was assayed in several independent experiments; representative single experiments are shown.
~1.8 M sucrose (Fig. 4). The \( M_r \) 16,000, 18,000, and 14,000 proteins were found in the same positions as before whereas the \( M_r \) 24,000 could not be detected, probably due to masking by other phosphoproteins (not shown). Marker enzyme analysis showed that essentially all of the ribosomes, mitochondria, intact lysosomes, and endoplasmic reticulum were shifted to a position at or near the bottom of the gradient (Fig. 5), most likely due to aggregation. A portion of the Golgi apparatus was also shifted to a higher density, whereas a substantial portion remained at the position seen with EDTA and EGTA. This eliminated the Golgi apparatus as the organelle containing the \( M_r \) 20,500 and 22,500 stimulus-affected phosphoproteins, because they were entirely shifted to the bottom of the gradient with Mg\(^{2+}\) and Ca\(^{2+}\). This leaves the endoplasmic reticulum as the only remaining location for these two proteins. The majority of the plasma membrane remained at 0.8–0.9 M sucrose with Mg\(^{2+}\) and Ca\(^{2+}\), as with EDTA and EGTA, eliminating plasma membrane as a possible location for the \( M_r \) 31,000 protein. This leaves the ribosomes and the outer mitochondrial membrane as the remaining possible locations for this protein.

The subcellular locations of the \( M_r \) 20,500, 22,500, and 31,000 would appear to argue against a direct role for these proteins in stimulus-secretion coupling, inasmuch as these organelles are not known to participate in exocytosis. The secretory granule location of the \( M_r \) 24,000 stimulus-affected phosphoprotein, however, appeared to make it an excellent candidate for a role in stimulus-secretion coupling, and so it was selected for more detailed study.

A procedure for obtaining highly purified rat parotid secretory granules was developed. This method was based on that developed by Castle et al. (52) for the purification of secretory granules from the rabbit parotid gland, but modified slightly since rat and rabbit parotid granules apparently differ somewhat in density (the unmodified rabbit parotid method gave very low yield when used with rat parotid glands [not shown]). Marker enzyme analysis showed that the secretory granules contained only very low levels of contaminating organelles, at or near the limits of biochemical detection (Table 1). Electron microscopy showed the presence of large numbers of typical electron-dense secretory granules with small amounts of amorphous material that may result from granule lysis during fixation (Fig. 6).

Analysis of granules purified from control, isoproterenol-stimulated, and carbamylcholine-stimulated \(^{32}P\)I-prelabeled tissue minces showed that the phosphorylation of the \( M_r \) 24,000 protein was reduced by carbamylcholine as well as by isoproterenol (Fig. 7). Carbamylcholine did not appear to affect the labeling of any other phosphoproteins. In contrast, isoproterenol increased the phosphorylation of several other secretory granule proteins, i.e., a doublet of ~\( M_r \) 92,500, a relatively weakly labeled band of ~\( M_r \) 19,000, and a very weakly labeled band of ~\( M_r \) 175,000. The stimulus-affected phosphoproteins in the purified secretory granule fraction appeared to be integral membrane proteins, because they were found in the particulate fraction when the granules were hypotonically lysed, and were not extracted from the pellet by 0.6 M KCl (Fig. 8). After the hypotonic lysis and KCl washing of the purified secretory granule fraction, amylase could not be detected enzymatically (although small amounts of the granule contents could be detected in silver-stained electrophoresis gels of the washed granule membrane fraction) (not shown). The wash procedure resulted in the disappearance of a large number of the nonstimulus-affected phosphoproteins.

**DISCUSSION**

The rat parotid phosphoprotein most obviously affected by stimulation, and hence the earliest to detect, is the \( M_r \) 31,000 protein whose phosphorylation is increased by isoproterenol. The \( M_r \) 31,000 described in this and our earlier studies (53, 54) is most probably the same protein described by other investigators as having a molecular weight of 27,000–35,000 (4, 6–10), and shown by Freedman and Jamieson (16) and Jahn and Söling (17) to be the ribosomal S6 phosphoprotein.

In previous studies using whole parotid homogenates we have described a \( M_r \) 19,000 protein whose phosphorylation was increased by isoproterenol (53, 54) and have found a protein with similar molecular weight and characteristics in our purified secretory granule preparation in this study. However, we have observed a major nonphosphorylated protein in parotid homogenates and secretory granule preparations of ~\( M_r \) 20,000, which is probably a granule content protein, and which distorts the position of proteins near it in electrophoresis gels. Therefore, the same phosphoprotein could appear to have different molecular weights in different subcellular fractions depending upon the presence or absence of this major granule content protein. We are therefore unsure whether the stimulus-affected phosphoprotein reported earlier in whole homogenates (53, 54) with an \( M_r \) of 19,000 is the same as the protein of this molecular weight found in the secretory granule membrane fraction or whether it corresponds to the \( M_r \) 20,500 protein of the endoplasmic reticulum.

We are similarly unsure whether the \( M_r \) 19,000 protein found in the secretory granule fraction is specific to the secretory granule fraction or whether it corresponds to the \( M_r \) 20,500 protein of the endoplasmic reticulum.
FIGURE 5 Distribution of subcellular markers in sucrose density gradients in the presence of Mg$^{2+}$ and Ca$^{2+}$. Conditions were as described in Materials and Methods. Results shown are compiled from several different experiments. Each marker was assayed in several independent experiments; representative single experiments are shown.
granule, or is due to a small amount of endoplasmic reticulum contaminating the granule fraction with the major nonphosphorylated granule content protein causing an apparent shift of the Mr 20,500 protein to 19,000. We are currently analyzing

| Subcellular organelle       | Marker used       | Recovery in purified granule fraction |
|-----------------------------|-------------------|--------------------------------------|
| Secretory granules          | a-Amylase         | 11.7                                 |
| Mitochondrial inner membrane| Succinate dehydrogenate | 1.5                                |
| Mitochondrial outer membrane| Monoamine oxidase | 0.1                                 |
| Plasma membrane             | y-Glutamyl transpeptidase | 1.5                                |
| Plasma membrane             | Alkaline phosphodiesterase | 0.9                                |
| Lysosomes                   | p-Glucuronidase   | 0.1                                 |
| Endoplasmic reticulum       | NADPH cytochrome c reductase | 1.1                                 |
| Golgi                       | Galactosyltransferase | 0.7                                 |
| Protein                     |                   | 4.6                                 |

Conditions were as described in Materials and Methods.

Several of the stimulus-affected phosphoproteins described in this study do not appear to have been previously reported. These include the Mr 18,000 cytoplasmic protein whose phosphorylation is decreased by isoproterenol, the Mr 24,000 secretory granule membrane phosphoprotein whose phosphorylation is decreased by both isoproterenol and carbamylcholine, and the Mr 175,000 and 92,500 doublet found in the purified secretory granule preparation whose phosphorylation was increased only by isoproterenol.

Although analysis of purified secretory granules revealed stimulus-affected phosphoproteins with molecular weights of 24,000, 92,500, and 175,000, only the Mr 24,000 protein was observed in the secretory granule peak in the continuous sucrose gradient fractionation of whole parotid homogenates, even when fractions were analyzed on 7% gels (not shown). The Mr 24,000 was more prominent than either the Mr 92,500 doublet or the Mr 175,000 protein in most preparations of purified secretory granules and therefore would be the easiest to detect in the gradient experiments. Because both autoradiography and marker analysis showed that purified secretory granules were less contaminated with other organelles than the secretory granule peak from the continuous gradients, the Mr 92,500 and 175,000 proteins may have been masked in

![Figure 6](http://example.com/figure6.png)  
**Figure 6**  
Electron micrograph of purified secretory granules from rat parotid gland. Granules were purified and prepared for microscopy as described in Materials and Methods. A representative field is shown. Sections from different parts of the fixed, pelleted granules showed no significant differences in granule morphology or presence of contaminants. × 12,500.
Figure 7 Autoradiogram showing effects of neurotransmitters on the degree of phosphorylation of secretory granule phosphoproteins. Secretory granules were purified from parotid minces labeled with $[^{32}P]P$, and either not stimulated (Cont.) or stimulated with 2 $\mu$M isoproterenol (Iso.) or 10 $\mu$M carbachol (Carb.). Purified granules were electrophoresed on 7 and 13% acrylamide slab gels. Arrowheads indicate stimulus-affected phosphoproteins. Experimental details are provided in Materials and Methods.

Our failure to detect the Mr 92,500 doublet and the Mr 175,000 protein in the gradient, and to show that their peak matches that of the secretory granules, makes it impossible to disprove the possibility that their presence in purified secretory granules is due to some other organelle contaminating these preparations. The high purity of these preparations appears to make this unlikely, however. One would expect that if these proteins were actually endogenous to the inner mitochondrial membrane, for example, they should be easily detectable in the gradients at 1.45 M sucrose (where this organelle peaks) because they were visible in purified secretory granules that contain only 1.5% of the total homogenate succinate dehydrogenase activity.

The methods used in this study can only describe and locate the stimulus-affected phosphoproteins, and not elucidate their function. However, the secretory granule membrane location of the Mr 24,000 phosphoprotein, and most probably of the Mr 175,000 protein and the Mr 92,500 doublet, suggests these phosphoproteins may participate in stimulus-secretion coupling. It is tempting to speculate that calcium mediates the effects of neurotransmitters on the dephosphorylation of the Mr 24,000 secretory granule membrane-associated phosphoprotein, because both carbamylcholine and isoproterenol produced this effect. Carbamylcholine is known to increase Ca$^{2+}$ influx into parotid minces whereas isoproterenol is known to mobilize intracellular Ca$^{2+}$ pools (reviewed in reference 55). The phosphorylation of the Mr 175,000 and 92,500 proteins may be regulated by cyclic AMP, because their phosphorylation is increased only by isoproterenol. If the observed alterations in granule membrane protein phosphorylation mediate exocytosis, such a regulatory scheme is able to account for much of what is presently known of the control of exocytosis in parotid. Carbamylcholine, for example, induces exocytosis because it, like isoproterenol, causes dephosphorylation of the Mr 24,000 protein, but is less effective than isoproterenol in stimulating exocytosis because it fails to stimulate the phosphorylation of the Mr 175,000 and 92,500 phosphoproteins. Similarly, the impaired ability of isoproterenol to induce exocytosis in Ca$^{2+}$-depleted tissue (55) may result from a failure to promote dephosphorylation of the Mr 24,000 phosphoprotein under these circumstances. Future experiments in this laboratory will be designed to test these hypotheses.

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