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

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ABSTRACT Rat parotid minces were labeled with $[^{32}P]P_i$, 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 (M_r 16,000 and 18,000) and increased the phosphorylation of a third (M_r 14,000). The phosphorylation of two endoplasmic reticulum proteins was increased by isoproterenol (M_r 20,500 and 22,500), as was an M_r 31,000 protein which was probably the S6 ribosomal protein. The phosphorylation of a secretory granule protein (M_r 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 M_r 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 cholinergic 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 $[^{32}P]P_i$, 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. $[\gamma\text{-}^{32}P]ATP$ was synthesized by the method of Johnson and Walseth (18) using enzymes from Boehringer Mannheim Bio-

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 OCTOBER 1984 1354–1363
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chemicals, Indianapolis, IN, and purified by the method of Palmer and Avruch (19). [α-^{32}P]ATP and [^{32}P]P_i were from New England Nuclear, Boston, MA. [^{3}H]Cyclic AMP and UDP-D-[6-^{3}H]galactose were from Amersham Corp., Arlington Heights, IL. Electrophoresis reagents, molecular weight markers, and dithiothreitol 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 KH_2PO_4 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 (HEPES-KOH), pH 7.4, 2 mM EDTA, and 0.2 mM ethyleneglycol-*bis* (β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) for each 0.1 g of tissue (wet wt). This medium has been reported to inhibit posthomogenization protein dephosphorylation with rat parotid (6). Connective tissue and unbroken cells were removed by centrifugation at 150 *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 containing 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM EGTA made in a 17 × 111-mm polypropylene centrifuge tube. Where indicated, the EDTA and EGTA was replaced with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in both the homogenizing medium and the gradient. The gradient was centrifuged at 4°C in a Beckman SW-28 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 *g* (max) for 16 h. The gradient was fractionated into ~0.5-ml fractions with a Buchler Auto Densi-Flow IIC (Searle Analytic, Inc., Fort Lee, NJ) and an ISCO Model 328 fraction collector (Instrumentation Specialties Co., Lincoln, NE). The sucrose concentration of each fraction was determined by refractometry (Bausch and Lomb, Rochester, NY).

PURIFICATION OF SECRETORY GRANULES: Parotid minces were prepared and homogenized as described above. The use of glass and nitrocellulose was avoided in all steps subsequent to tissue homogenization and all solutions used in the purification of secretory granules contained 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM EGTA. Unbroken cells and connective tissue were removed by centrifugation as before and the supernatant was layered onto a discontinuous sucrose density gradient using 3 ml of 1.0 M sucrose over 3 ml of 2.0 M sucrose. The gradients were centrifuged for 20 min at 1,700 *g* (max) (4°C) (IEC Centra-7R). The crude granules were collected at the interface between 1.0 and 2.0 M sucrose. The sucrose concentration of the crude granules was adjusted to 1.5 M, and they were layered onto a second discontinuous gradient composed of 2 ml of 1.7 M sucrose over 3.5 ml of 2.0 M sucrose. The granules were overlaid with 3.5 ml of 1.0 M sucrose and centrifuged 90 min at 150,000 *g* (max) (4°C) (SW-41 rotor, Beckman Instruments, Inc.). Purified secretory granules were collected at the 1.7–2.0 M interface and were used for electron microscopy or assayed for subcellular organelle marker activities without further processing. When the granules were to be analyzed by electrophoresis or used to prepare a secretory granule membrane fraction, they were concentrated by centrifugation (60 min, 150,000 *g* (max), 4°C, Beckman Ti50) after dilution to 0.3 M sucrose with 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM EGTA.

PREPARATION OF SECRETORY GRANULE MEMBRANES: Parotid secretory granules were prepared and concentrated as described above. The final pellets were resuspended in 9 ml of 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM EGTA and recentrifuged (60 min, 150,000 *g* (max), 4°C, Beckman Ti50 rotor). The pellets were resuspended in 8 ml of 0.6 M KCl, 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, 0.2 mM EGTA, and centrifuged as above. The resulting pellet was used as the secretory granule membrane fraction.

ENDOGENOUS PROTEIN LABELING: Each sample consisted of minced parotid tissue equivalent to two glands, and was divided into two approximately equal portions. Endogenous parotid phosphoproteins were prelabeled by incubating each portion in 1 ml of incubation media containing 0.3 mCi [^{32}P]P_i at 37°C under an O_2 atmosphere for either 1 h (continuous sucrose density-gradient experiments) or 3 h (secretory granule purification experiments). All incubations were performed in 20-ml plastic scintillation vials. Prelabeled minces were then stimulated with 2 μM isoproterenol or 10 μM carbamylcholine for 10 min as indicated in the legends to individual figures. Incubations were terminated by combining the two halves of each sample on a piece of polypropylene gauze stretched over a beaker and washing extensively with homogenizing medium. The tissue was immediately transferred to a glass homogenizer and homogenized as described above.

ELECTROPHORESIS AND AUTORADIOGRAPHY: In continuous sucrose density-gradient experiments, 50 μl of each fraction was combined with 50 μl of electrophoresis stopping solution (0.1 M Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, and 20% glycerol) and boiled 3 min. 50-μl aliquots were electrophoresed on 13% SDS polyacrylamide slab gels according to Laemmli (21).

In experiments with purified secretory granules or secretory granule membranes, the final pellets were resuspended in 35 μl 0.3 M sucrose, 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 0.2 mM EGTA. An equal volume of electrophoresis stopping solution was added and the samples were boiled for 3 min. The radioactivity of each sample was determined by counting 5-μl aliquots in 4 ml of Aqueous Counting Scintillant (ACS, Amersham Corp.). The samples were diluted so that equal volumes of different samples contained the same number of counts and 50-μl aliquots were electrophoresed on 7 and 13% SDS polyacrylamide slab gels according to Laemmli (21).

Gels were stained using the method of Sammons et al. (22), equilibrated in 5% methanol, 7.5% acetic acid, and dried using a Bio-Rad model 224 Gel Slab Dryer (Rockville Center, NY). The radioactivity contained within each gel was estimated with a geiger counter and each gel was exposed to Kodak NST x-ray film (Picker Corporation, Pittsburgh, PA) for the length of time empirically determined to be optimal for the detection of neurotransmitter effects on phosphoprotein labeling.

CALCULATIONS: Autoradiograms from continuous sucrose density-gradient experiments were scanned on a Zeineh Soft Laser Scanning Densitometer, model SL-504-XL (Biomed Instruments Inc., Fullerton, CA). The integrated areas of the isoproterenol-affected phosphoproteins in each fraction were corrected for slight differences in the amount of total protein between 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 100 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 $MnCl_2$, 1 mM dithiothreitol, 200 μg of sialic acid-free, galactose-free fetuin, 50 μM UDP-D-[6-^{3}H]galactose (2 μCi/mmol), 0.75% Triton X-100, and sample in a final volume of 60 μl. Samples consisted of 10 μl of each fraction preactivated 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 from the disks, the retained radioactivity was solubilized with H_2SO_4 (24) and counted in 3 ml of ACS.

Succinate 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 Greenawalt (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 Kanarek 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 transpeptidase was assayed using γ-glutamyl-*p*-nitroanilide 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). Duplicate 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_i 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 amylase, 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]P_i from [γ-^{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 $MgSO_4$, and 2 mM [γ-^{32}P]ATP (0.25 mCi/mmol) 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 KH_2PO_4. The charcoal was removed by centrifugation and 750 μl of the supernatant counted in 3.5 ml of ACS.

Rotenone-insensitive NADH cytochrome *c* reductase was assayed by a modification 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 cytochrome *c*, 0.1 mM NADH, 2 μM 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 $MgCl_2$, 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 centrifugation and the P_i content of 200 μl of the supernatant was determined by the method of Chen et al. (34).

Nucleoside diphosphatase was assayed by a modification of the method of Beaufay et al. (40). The assay contained 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 2.5 mM IDP, and 40 μl of sample in a final volume of 200 μl. Duplicate aliquots of each fraction were assayed with one aliquot boiled before assay to serve as a blank. The incubation was for 30 min at 37°C, and was terminated by boiling for 3 min. 1 ml of ice-cold 10 mg/ml charcoal was added to the cooled samples. The charcoal was removed by centrifugation, and the P_i content of the supernatant was determined on a 700-μl aliquot using the method of Chen et al. (34).

Thiamine pyrophosphatase was assayed by the method of Meldolesi 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_i 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. Fractions 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% OsO_4 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 ultramicrotone (Newton, CT), stained with 3.0% (wt/vol) uranyl acetate and Reynold's lead citrate (42). Microscopy was performed 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 phosphoproteins 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. Isoproterenol 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 M_r 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 M_r 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. 2*c*). The M_r 31,000 phosphoprotein was highly labeled and widely distributed, with a peak at 0.8–0.9 M sucrose (Fig. 2*d*). The M_r 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 M_r 24,000 protein was found near the bottom of the gradient at a sucrose concentration of ~1.7–1.8 M (Fig. 2*g*).

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 dehydrogenase, a cytoplasmic marker (43), appeared at the top of the gradient, peaking at 0.3–0.4 M sucrose (Fig. 3*a*). The plasma membrane markers adenylate cyclase (43), 5′-nucleotidase (44), Na/K ATPase (43), γ-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. 3*h*). β-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. 3*i*). The heavier peak probably represents intact lysosomes whereas the higher peak may result from β-glucuronidase released from lysosomes ruptured during tissue homog-

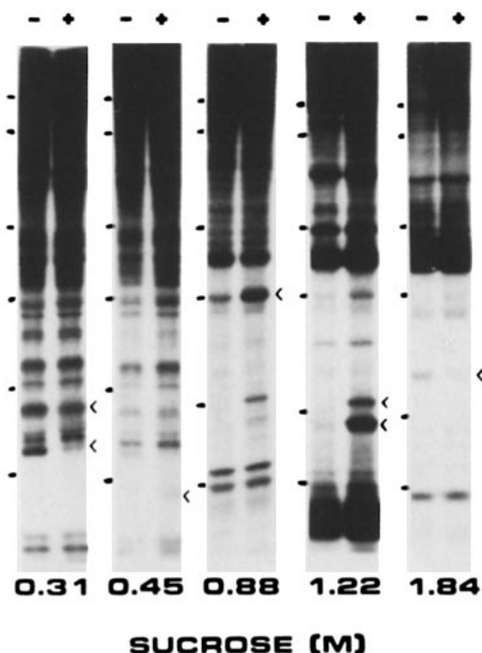

FIGURE 1 Autoradiogram showing the endogenous rat parotid phosphoproteins affected by isoproterenol detected in sucrose gradients in the presence of EDTA and EGTA. Parotid minces were prelabeled with [^{32}P]P_i, incubated with (+) or without (−) 2 μM isoproterenol, homogenized, fractionated on sucrose gradients, and analyzed by PAGE as described in Materials and Methods. Selected slots from a representative experiment are shown. Stimulus-affected phosphoproteins are indicated by arrowheads to the right of each pair of tracks. The approximate positions of molecular weight markers are indicated to the left of each pair of tracks. The markers used were (from top) phosphorylase *b* (92,500 mol wt), BSA (66,200 mol wt), ovalbumin (45,000 mol wt), carbonic anhydrase (31,000 mol wt), soybean trypsin inhibitor (21,500 mol wt), and lysozyme (14,400 mol wt).

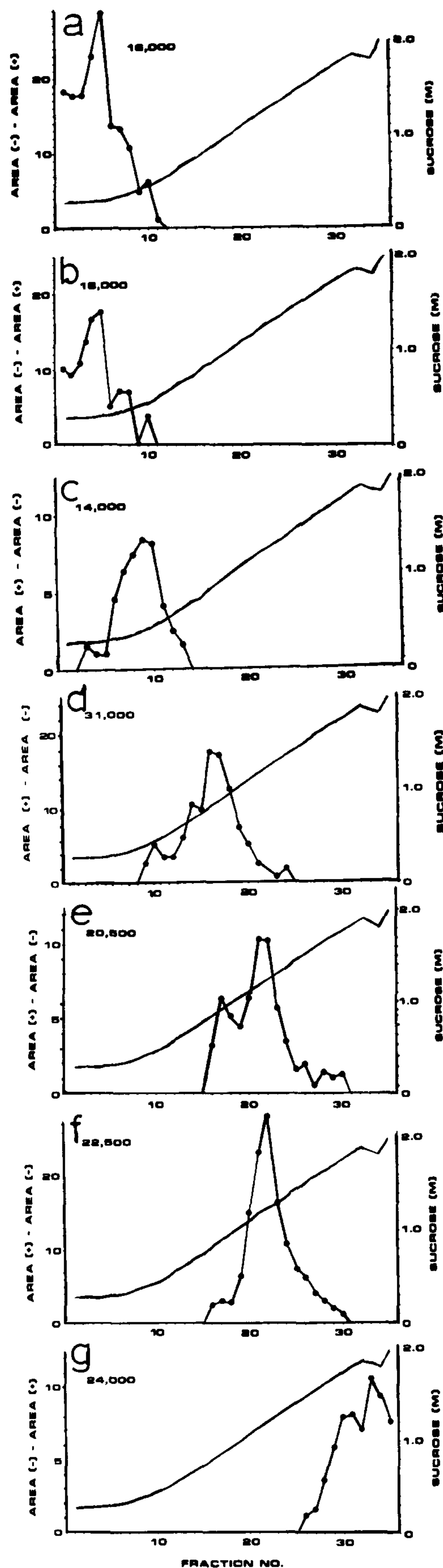

FIGURE 2 Distribution of isoproterenol-affected rat parotid endogenous phosphoproteins in sucrose density gradients in the presence of EDTA and EGTA. Conditions were as described in Materials and Methods. Results shown are from a single representative experiment.

enization. Amylase, a marker for secretory granules in parotid (46), also showed a bimodal distribution with peaks at 0.3–0.4 M and 1.7–1.8 M sucrose (Fig. 3*j*). As before, the heavier peak probably represents intact granules while the lighter peak represents the contents of granules released from granules ruptured during homogenization. Both Golgi markers, galactosyltransferase (46) and thiamine pyrophosphatase, exhibited peaks at 1.1–1.2 M sucrose, whereas thiamine pyrophosphatase exhibited an additional peak at 0.3–0.4 M sucrose (Fig. 3, *k* and *l*), which is probably due to non-Golgi pyrophosphatase activity. Nucleoside diphosphatase has been reported to reside in the plasma membrane in some tissues (48) and the endoplasmic reticulum (49) in others. In rat parotid it is apparently in the plasma membrane because it peaked with the other plasma membrane markers at ~0.9 M sucrose (Fig. 3*m*), whereas endoplasmic reticulum markers NADPH cytochrome *c* reductase (29) and glucose-6-phosphatase (47) both peaked at 1.1–1.2 M sucrose (Fig. 3, *n* and *o*).

It appeared as though the homogenization and/or fractionation procedures used in this study stripped the outer membrane from the mitochondria in that the outer mitochondrial membrane marker monoamine oxidase (26) peaked at 0.8–0.9 M sucrose (Fig. 3*q*), whereas the inner mitochondrial membrane marker succinate dehydrogenase (26) to the matrix enzyme fumarase (50) showed peaks at 1.4 and 1.5 M sucrose (with fumarase showing an additional minor peak at 0.8–0.9 M) (Fig. 3, *p* and *r*). Adenylate kinase, reported to reside in the space between the inner and outer mitochondrial membranes (26) peaked at 0.3–0.5 M sucrose with a second peak at 1.4–1.5 M (Fig. 3*s*). Rotenone-insensitive NADH cytochrome *c* reductase, reported to reside in both the outer mitochondrial membrane (26) and the endoplasmic reticulum (51), showed peak activity at 1.1–1.2 M sucrose, with a shoulder of activity at ~0.8–0.9 M (Fig. 3*t*).

Electron microscopy of pooled fractions from the sucrose gradient confirmed the results of the marker enzyme analysis (not shown). Fractions containing from 0.8 to 1.0 M sucrose showed the presence of membranes and ribosomes, whereas fractions containing 1.1–1.3 M sucrose consisted mostly of membranes with occasional small mitochondria and secretory granules (not shown). Fractions containing 1.4–1.55 M sucrose were highly enriched in mitochondria that were rounded and appeared to lack their outer membrane. Fractions containing 1.75–1.85 M sucrose consisted of secretory granules with only an occasional mitochondrion present.

Based on these results using sucrose gradients containing EDTA and EGTA, it appeared as though the M_r 14,000, 16,000, and 18,000 proteins were cytoplasmic while the M_r 31,000 protein was either in the ribosomes, the plasma membrane, or the outer mitochondrial membrane. The M_r 20,500 and 22,500 appeared to be associated with either the endoplasmic reticulum or the Golgi apparatus. The M_r 24,000 protein appeared to be associated with the secretory granules.

We found that, by replacing the EDTA and EGTA with Mg^{2+} and Ca^{2+}, some of the stimulus-affected phosphoproteins and subcellular organelle markers were shifted to new positions in the gradient, thereby affording the opportunity to further define the subcellular localization of the stimulus-affected phosphoproteins. The M_r 31,000, 20,500, and 22,500 proteins were all found near the bottom of the gradient at

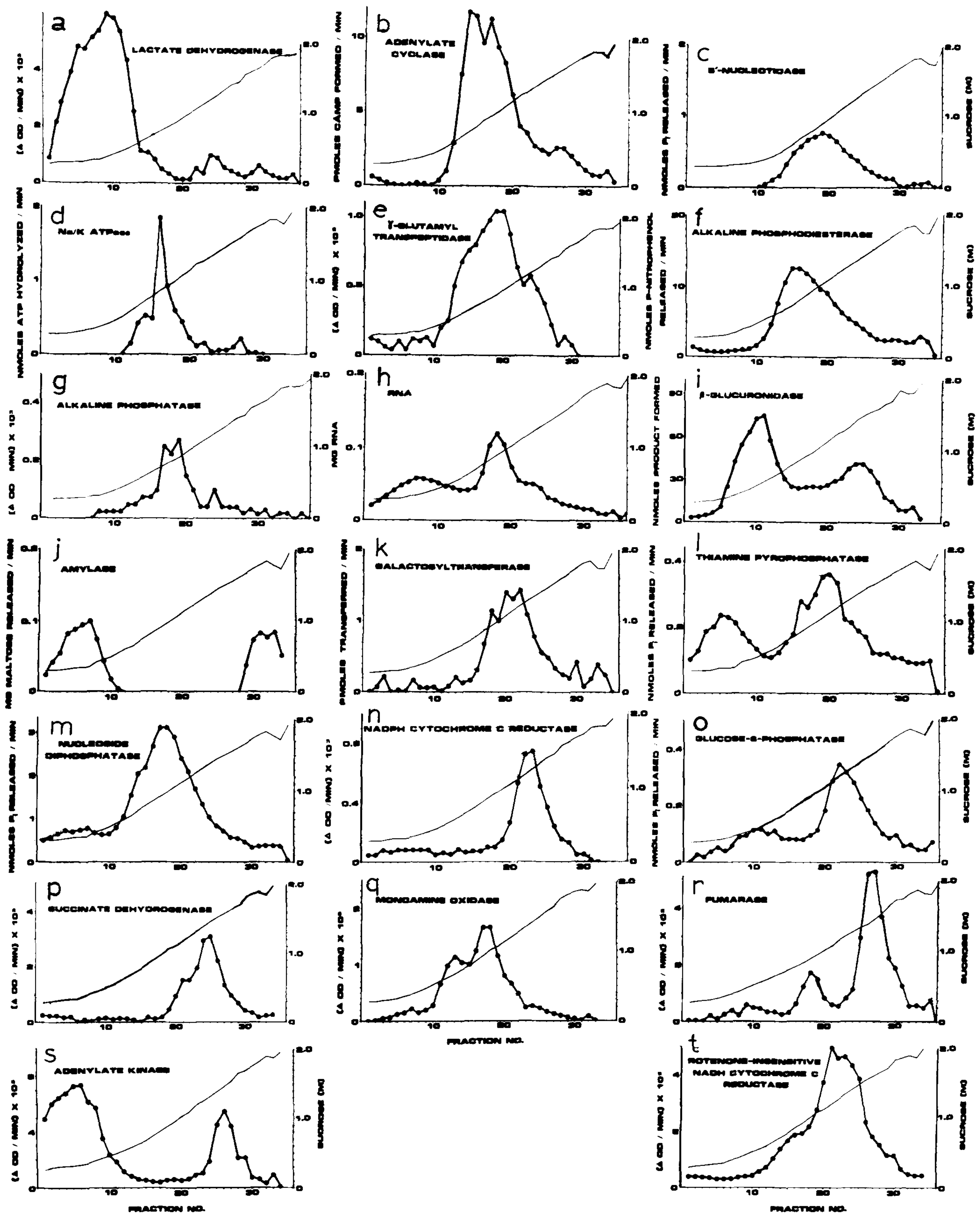

FIGURE 3 Distribution of subcellular markers in sucrose density gradients in the presence of EDTA and EGTA. Conditions were as described in Materials and Methods. Results shown are compiled from several different experiments. Each marker was assayed in several independent experiments; representative single experiments are shown.

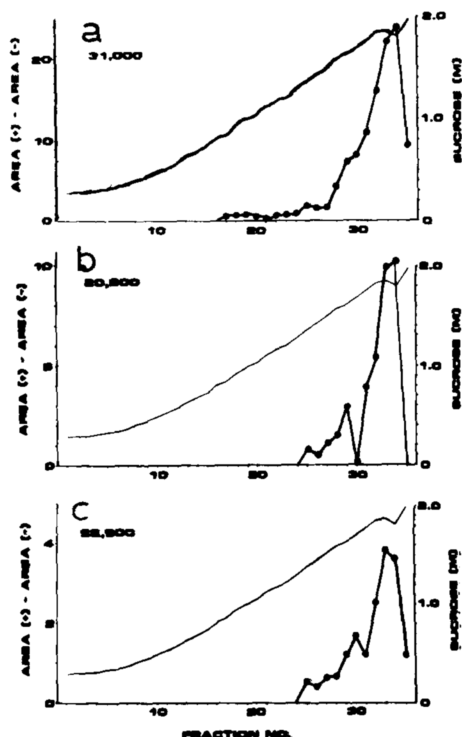

FIGURE 4 Distribution of isoproterenol-affected rat parotid endogenous phosphoproteins in sucrose density gradients in the presence of Mg^{2+} and Ca^{2+}. Conditions were as described in Materials and Methods. Results shown are from a single representative experiment.

~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 I). 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 $[^{32}P]P_i$-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

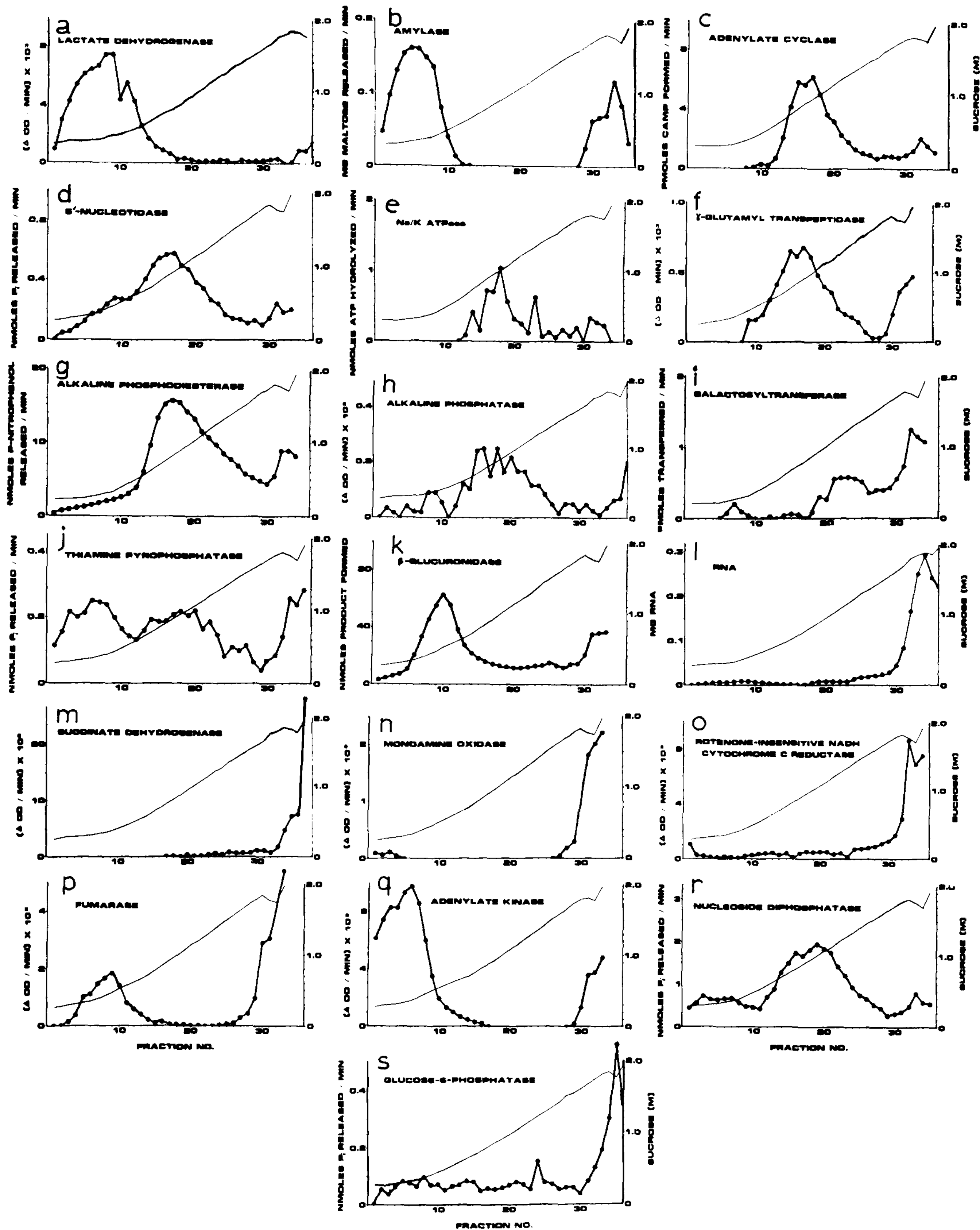

FIGURE 5 Distribution of subcellular markers in sucrose density gradients in the presence of Mg^{2+} and Ca^{2+}. Conditions were as described in Materials and Methods. Results shown are compiled from several different experiments. Each marker was assayed in several independent experiments; representative 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 M_r 20,500 protein to 19,000. We are currently analyzing purified secretory granules and an endoplasmic reticulum-enriched fraction by two-dimensional electrophoresis in an attempt to resolve this question.

TABLE I

Marker Analysis of Purified Secretory Granules

Subcellular organelle	Marker used	Recovery in purified granule fraction
		%
Secretory granules	α-Amylase	11.7
Mitochondrial inner membrane	Succinate dehydrogenate	1.5
Mitochondrial outer membrane	Monoamine oxidase	0.1
Plasma membrane	γ-Glutamyl transpeptidase	1.5
Plasma membrane	Alkaline phosphodiesterase	0.9
Lysosomes	β-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 M_r 18,000 cytoplasmic protein whose phosphorylation is decreased by isoproterenol, the M_r 24,000 secretory granule membrane phosphoprotein whose phosphorylation is decreased by both isoproterenol and carbamylcholine, and the M_r 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 M_r 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 M_r 24,000 was more prominent than either the M_r 92,500 doublet or the M_r 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 M_r 92,500 and 175,000 proteins may have been masked in

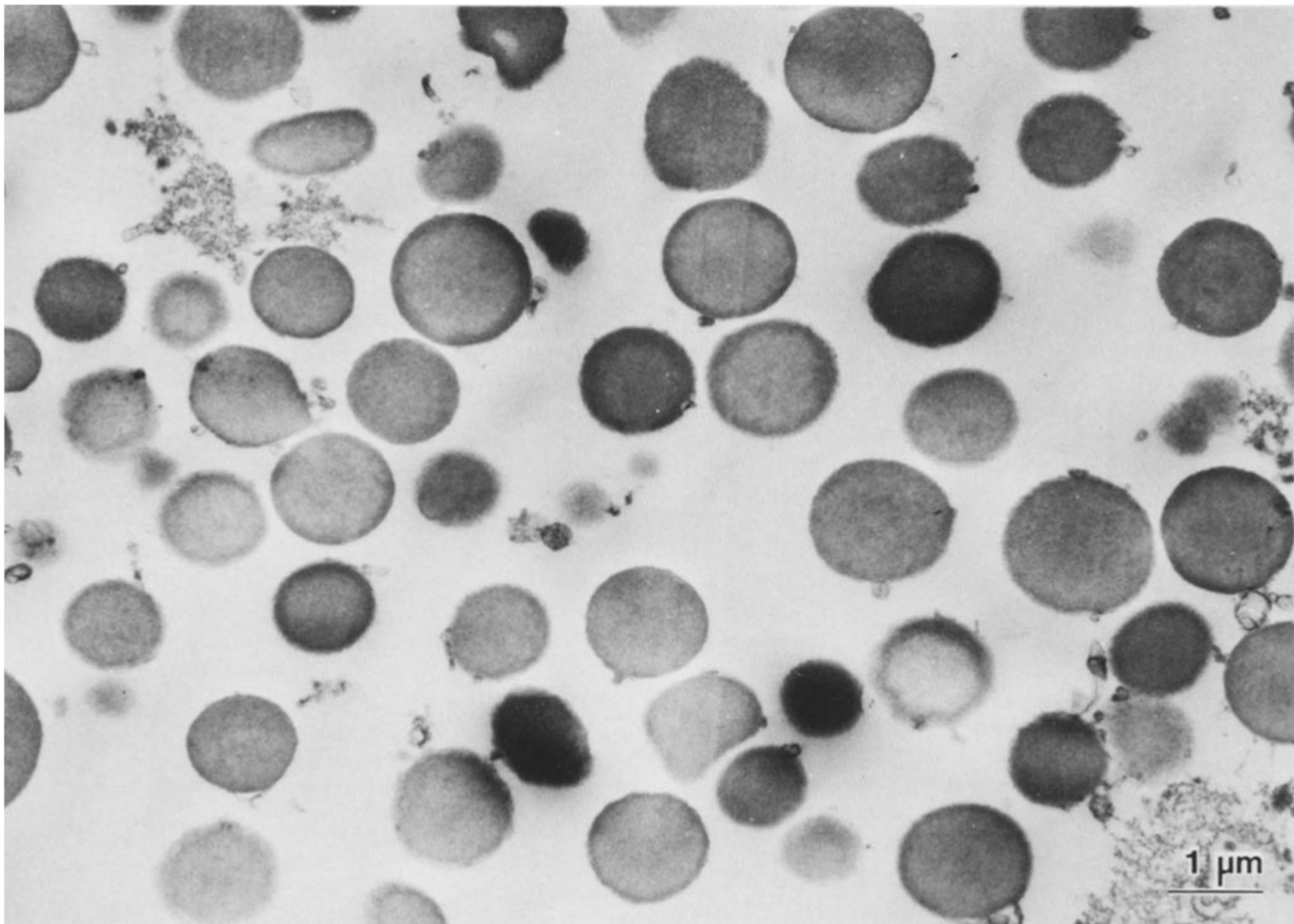

FIGURE 6 Electron micrograph of purified secretory granules from rat parotid gland. Granules were purified and prepared for microscopy as described in Materials and Methods. A representative field is shown. Sections from different parts of the fixed, pelleted granules showed no significant differences in granule morphology or presence of contaminants. × 12,500.

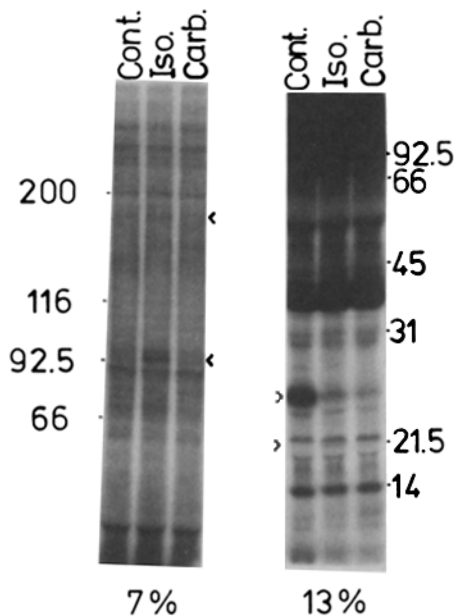

FIGURE 7 Autoradiogram showing effects of neutrotransmitters on the degree of phosphorylation of secretory granule phosphoproteins. Secretory granules were purified from parotid minces labeled with [^{32}P]P$_i$ and either not stimulated (*Cont.*) or stimulated with 2 μM isoproterenol (*Iso.*) or 10 μM 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.

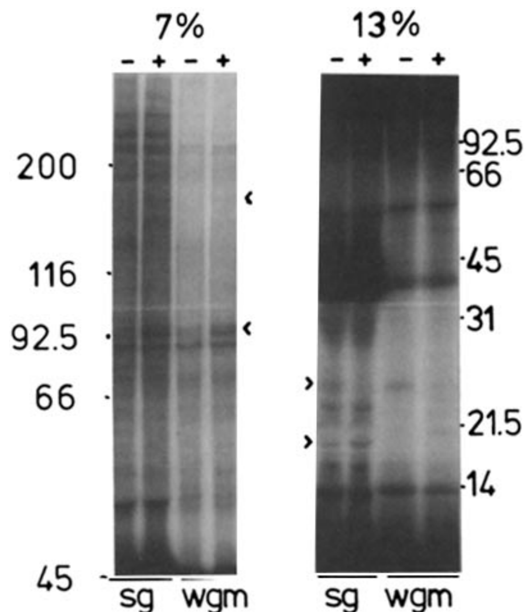

FIGURE 8 Autoradiogram showing the effect of isoproterenol on the phosphorylation of secretory granule and secretory granule membrane phosphoproteins. Parotid minces were labeled with [^{32}P]P$_i$ and either not stimulated (−) or stimulated with 2 μM isoproterenol (+) and the secretory granules were purified. An aliquot of the secretory granules (*sg*) was lysed and a KCl-washed granule membrane fraction prepared (*wgm*). The granules and washed granule membranes were electrophoresed on 7 and 13% acrylamide slab gels. Arrowheads indicate stimulus-affected phosphoproteins. Experimental details are provided in Materials and Methods. The secretory granules contain a major nonphosphorylated phosphoprotein which distorts the position of the M_r 19,000 protein in gel. This nonphosphorylated protein is removed during the granule lysis and wash procedure, leading to an apparent difference in the molecular weight of the M_r 19,000 protein in the secretory granule fraction and the secretory granule membrane fraction.

the gradients by more highly labeled co-migrating phosphoproteins from other organelles.

Our failure to detect the M_r 92,500 doublet and the M_r 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 M_r 24,000 phosphoprotein, and most probably of the M_r 175,000 protein and the M_r 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 M_r 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 M_r 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 M_r 24,000 protein, but is less effective than isoproterenol in stimulating exocytosis because it fails to stimulate the phosphorylation of the M_r 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 M_r 24,000 phosphoprotein under these circumstances. Future experiments in this laboratory will be designed to test these hypotheses.

We thank Dr. J. P. Durham, Departments of Biochemistry and Surgery, Dr. H. A. Thompson, Department of Microbiology, and Dr. J. S. Fedan, Department of Pharmacology, West Virginia University, for their recommendations concerning the assay of several of the marker enzymes used in this study.

This work was supported by a New Investigator Award to Dr. Spearman from the Cystic Fibrosis Foundation and a grant from the National Institutes of Health (AM-25249). Kevin P. Hurley was supported by a Cystic Fibrosis Foundation Student Traineeship.

Received for publication 5 July 1983, and in revised form 12 June 1984.

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