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PHOSPHORYLATION OF RABBIT PAROTID MICROSOMAL PROTEIN OCCURS ONLY WITH β-ADRENERGIC STIMULATION

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SUMMARY

β-adrenergic stimulation of rabbit parotid gland secretion is associated with phosphorylation of a 34,000 (major) and a 30,000 (minor) molecular weight protein located in the microsomal fraction. Phosphorylation of the same proteins is not observed with either carbachol or phenylephrine despite the fact that both stimulate amylase release. The evidence is consistent with the hypothesis that phosphorylation of specific proteins in the plasma membrane is involved in β-adrenergic stimulated exocytosis.

Stimulation of the parotid glands with β-adrenergic agonists leads to a large increase in intracellular cyclic AMP (1,2). It has been proposed that cyclic AMP mediates the effect of β-adrenergic stimulation on protein secretion (determined by amylase release)(3). Further credence has been given to this proposal by two recent findings. One, cyclic AMP-dependent protein kinase (cAMP-PK)[2] is activated by β-adrenergic stimulation (4). Dose response and time course studies of cAMP-PK activation are consistent with its proposed role in the secretory process. Two, the phosphorylation of a 30,000-35,000 molecular weight protein has been shown to be stimulated by β-adrenergic agonists (5,6). The β-adrenergic-stimulated phosphorylation appears to occur in the subcellular fractions enriched in plasma membranes (5).

This paper examines the specificity of phosphorylation of a 34,000 and a 30,000 molecular weight protein in a microsomal fraction from isolated rabbit

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[2] Abbreviations: cAMP-PK, cyclic AMP-dependent protein kinase; MIX, 3-isobutyl-1-methylxanthine; cAMP-PK_I and cAMP-PK_{II}, isozymes of cAMP-PK; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N-N'tetraacetic acid; EDTA, ethlenediaminetetraacetic acid; PIPES, piperazine-N-N'-bis[2-ethane sulfonic acid].

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parotid acinar clumps in the presence of the β-adrenergic agonist, isoproterenol. In addition, the effects of cholinergic (muscarinic) and α-adrenergic stimulation of phosphorylation and amylase release are examined. The latter agents have been reported to produce significant amylase secretion without appreciable increases in cyclic AMP (2,3,7,8). These drugs are shown here not to enhance the phosphorylation of the proteins at 34,000 and 30,000 molecular weight as is the case for β-adrenergic stimulation.

METHODS AND MATERIALS

Isolation and incubation of parotid cell clumps. Rabbit parotid acinar clumps were isolated from New Zealand white rabbits by a modified method of Kanagasuntheram and Randle (9). The isolation was performed in Krebs Ringer bicarbonate solution containing 5 mM β-hydroxybutyrate (KRB). The cells (250-350 mg wet weight) were then suspended in 2.67 ml of 5 mM Hepes solution (pH 7.1-7.4), containing 118 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 14 mM glucose, 1% bovine serum albumin (BSA) with 1.0 mCi of carrier free inorganic ^{32}P (10). After one hour of incubation at 37°C with shaking, the cells were spun at 300 g for 3 minutes and the supernatent aspirated off. Washing of the cells was conducted accordingly (10).

Washed cells were then suspended in KRB at a concentration of 40-60 mg wet wt/ml. Cells (1.4 ml) were then added to siliconized test tubes and incubated with drugs as indicated (9) except that 5 mM β-hydroxybutyrate, 1.28 mM $CaCl_2$, and 1.0% BSA were present in all solutions. Tubes were gassed every 10-15 minutes with 95% O_2-5% CO_2. Drugs were prepared in KRB and added in one-tenth the assay volume. MIX (10 μM) was included with all drugs since preliminary studies showed that it enhanced the response of isoproterenol-induced cAMP-PK activation. MIX (10 μM) alone did not stimulate cAMP-PK. Ascorbic acid (0.1 mM) was also included to prevent non-enzymatic breakdown of isoproterenol. Control tubes were treated likewise. After incubation, the cells were centrifuged at 13,500 RPM for 30 seconds. A 50 μl portion of supernatent was removed for measurement of release amylase activity. The remainder of the supernatent was discarded. The centrifuged cells were immediately suspended in 3.0 ml of 0.28 M sucrose, 40 mM Tris (pH 7.2), 0.2 mM EDTA, 10 mM KF at 4°C, and homogenized using 12 complete strokes with a motor-drive pestle. A 175,000 g pellet (microsomal fraction) was obtained after the cells were fractionated and the homogenate precentrifuged at 600 g (max.) (8 minutes) and then 10,000 g (max.) (15 minutes). Lack of sufficient membrane material prevented further subfractionation of the microsomal fraction as it was in a previous study (11). Enzymatically the microsomal fraction was shown to be enriched in glucose-6-phosphatase (2.5 fold), Na^+,K^+-ATPase (6 fold) and alkaline phosphatase (1.5 fold) compared to activity in the tissue homogenate (11). The pellets were dissolved in 2% SDS, 0.0625 M Tris-HCl (pH 6.8), 18% glycerol, 12 mM dithiothreitol by heating for 3 minutes in boiling water. All proteins, including protein standards, were dialyzed against the same buffer. Protein in each fraction was estimated by absorption at 280 nm prior to electrophoresis using BSA as a standard and ranged from 1 to 3 mg/ml. Equivalent amounts of radioactivity in control and drug-treated samples were applied to the gels.

Discontinuous SDS polyacrylamide gel electrophoresis - Autoradiography. Slab gels (1.5 mm thick) and autoradiography were used to identify phosphorylated protein peaks. The system of Laemmli (12) was used except that a 5-15% acrylamide gradient was employed as the separating gel with a 3% acrylamide stack-

ing gel. Gels were stained, then destained and dried. Autoradiography was performed by placing a Kodak X-Omat AR 5"x7" x-ray film on top of the dried gel for a period of 24-70 hours. Tube gels were also run by utilizing the method of Laemmli (12). These gels were sliced for quantitating phosphorylation of specific proteins by liquid scintillation counting. In addition. autoradiographs were scanned.

Determination of amylase activity and protein. Amylase was measured from aliquots of the supernatents by the method of Bernfeld (13). Protein was determined by the method of Lowry, et al. (14), after washing the cells to remove BSA. Amylase release from cells is reported in percentage of total cell content of amylase.

Preparation of a plasma membrane fraction from whole glands. Routinely, 16 glands were used for membrane isolation. Details of the isolation procedure have been published (11). Plasma membrane-enriched particles were isolated from a 140,000 g (max.) pellet by using a discontinuous sucrose gradient composed of 0.90 M and 1.15 M sucrose. Each layer contained 40 mM imidazole (pH 7.4), 1 mM EDTA. Plasma membranes, characterized by Na^+,K^+-ATPase specific activity, were collected at the 0.90-1.15 M sucrose interface. The membrane fraction was diluted to 0.28 M sucrose and centrifuged at 175,000 g (max.). Membranes were resuspended in 30 mM imidazole (pH 7.4), 1 mM EDTA at a concentration of 0.75-2 mg protein/ml for phosphorylation studies.

Phosphorylation of isolated plasma membranes from whole glands. The membrane phosphorylation reactions were carried out in 50 mM PIPES buffer (pH 6.6) containing 0.2 mM (γ^{32}P)-ATP (14 μCi), 4 mM magnesium acetate, 10 mM KF, 0.5 mM MIX, 0.3 mM EGTA, 360 μg of membranes and 150 μg of protein kinase with or without 5 μM cyclic AMP. Membranes were heat-treated for 20 minutes at 90°C prior to phosphorylation. cAMP-PK fractions were isolated from the cytosolic fraction of whole rabbit parotid glands by DEAE cellulose chromatography (15). Isozyme II of cAMP-PK was used for this study even though cAMP-PK_I gave similar results. Control experiments indicated that protein phosphorylation as seen in our studies was not due to the phosphorylation of proteins in the exogenously added protein kinase.

Chemicals. (γ^{32}P)-ATP and (^{32}P)-Pi were purchased from New England Nuclear. dl-isoproterenol, carbachol, l-phenylephrine, dl-propranolol and MIX were purchased from Sigma. Chemicals were reagent grade or better.

RESULTS AND DISCUSSION

Amylase Secretion. Table I shows that all three agonists, isoproterenol (a β receptor agonist), carbachol (a muscarinic receptor agonist) and phenylephrine (an α receptor agonist), stimulated amylase release from the cells. Propranolol (20 μM) was included to eliminate β adrenergic stimulation when carbachol and phenylephrine were used. Similar reports have been published indicating that all three agents stimulate secretion of amylase (2,7,8). It has been determined, however, that β-adrenergic stimulation in the parotid gland is distinct in its mechanisms from the other two (2,7,8). Furthermore, only β-adrenergic stimulation increases cyclic AMP concentrations significantly in the parotid gland (2,7,8).

Protein Phosphorylation Studies. We examined the effect of the three autonomic drugs on phosphorylation in intact acinar cell clumps from the parotid

TABLE I

Effect of Autonomic Agents on Amylase Release
From Acinar Cell Clumps from Rabbit Parotid Gland

CONDITION	TIME (MIN.) 10	30
Control	6.9±1.1	6.6±2.2
Isoproterenol (4 μM)	16.6±1.5	24.2±4.6
Control	5.9±0.5	7.1±1.0
Carbachol (10 μM)	9.9±1.4	13.8±0.8
Control	7.5±0.6	
Phenylephrine (25 μM)	11.4±1.2	

Cell supernatent fractions were analyzed as described in Methods. Isoproterenol incubations were performed in the presence of 10 μM MIX. Carbachol and phenylephrine incubations were performed in the presence of 10 μM MIX plus 20 μM propranolol. Values are means ±S.E.M. from 4-6 experiments.

gland. Figure 1 shows that by using slab gel electrophoresis, the phosphorylation of two distinct protein bands is stimulated in the presence of isopro-

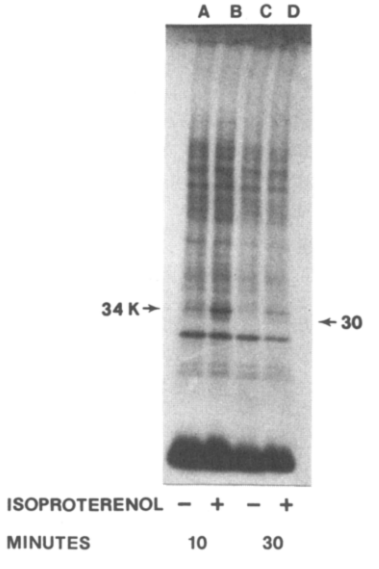

FIGURE 1: Autoradiograph of SDS-acrylamide gel. Incubations with isoproterenol (4 μM) were conducted for 10 and 30 minutes. Cells were then fractionated and 175,000 g fractions were dissolved in SDS solution as described in Methods. Sixteen μg of protein was applied to wells A and B and 14 μg protein applied to wells C and D. Isoproterenol-stimulated phosphorylations are indicated by arrows at 34,000 and 30,000. The dye front is the bottom edge of the gel.

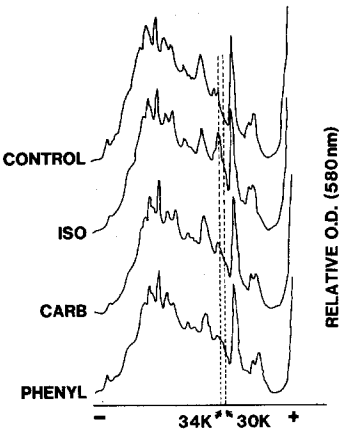

FIGURE 2: Comparison of autoradiographs of autonomic agents. Drug protocols are listed in the text. Dashed lines (34,000 and 30,000 M.W.) indicate proteins whose phosphorylation is enhanced by isoproterenol. Data represents drug incubations of 10 minutes. Isolation of the microsomal fraction from isolated cell clumps was conducted as described in Methods except 5 μM phenylmethylsulfonyl fluoride and 5 μM benzamine were included in the cell homogenization step to inhibit potential endogenous protease activity. The dye front is at the right.

terenol. The apparent molecular weights of these bands in our hands were 34,000 and 30,000. Recent evidence from other laboratories resembles these findings. Jahn, et al. (5) and Kanamori and Hayakawa (6) have shown stimulation of phosphorylation by isoproterenol of one 30,000-35,000 molecular weight protein in plasma membranes from rat parotid slices. Similar findings were found in the rat parotid (16,17,18) and in the rat submandibular gland (19).

We further examined the effects of the non-β adrenergic agonists on phosphorylation of the 34,000 and 30,000 molecular weight proteins. Neither carbachol (10 μM) nor phenylephrine (25 μM) stimulated phosphorylation of these proteins during 10 minutes (Fig. 2) or 30 minutes (not shown) of drug incubation, even though at these time periods both agents stimulated amylase release.

We next examined the protein phosphorylation profile of an isolated plasma membrane-enriched fraction taken from whole glands. The membranes derived from whole glands differed from those derived from the isolated cell clumps

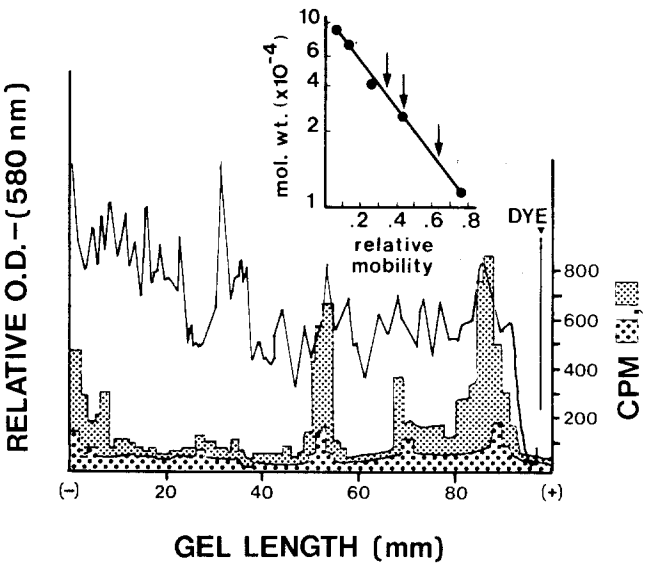

FIGURE 3: SDS-polyacrylamide disc gel electrophoretic profile of parotid plasma membranes from whole glands. Phosphorylation was conducted for 30 minutes with exogenously added rabbit parotid cAMP-PK_{II} and heat-treated membranes. Samples were prepared for electrophoresis and electrophoresis was conducted as described in Methods except that 10% polyacrylamide was used as the separation gel. CPM = counts per minute in presence (area with small dots) or absence (area with large dots) of cyclic AMP. Profile is representative of three differenet membrane preparations. Inset is the standard curve: phosphorylase, 93,000; BSA, 68,000; aldolase, 40,000; chymotrypsinogen, 25,700; cytochrome C, 11,700. Arrows indicate mobility of major ^{32}P-incorporated proteins.

in that the former were more enriched in Na^+,K^+-ATPase activity (11) and that they were heat-treated to reduce endogenous ATPase activity prior to phosphorylation. The phosphorylation of three low molecular weight substrates in whole cell plasma membranes was stimulated by cyclic AMP (5 μM) (36,000, 25,000 and 17,000 M.W.) (Fig. 3). The 36,000 M.W. protein was similar in mobility to the 34,000 M.W. isoproterenol-stimulated protein (see above). They may represent the same cAMP-PK substrate. If so, the isolated plasma membrane experiment supports the plasma membrane authenticity of the 34,000-36,000 M.W. protein. It is not known yet why the other two smaller molecular weight substrates were not stimulated by isoproterenol in intact isolated cells as they were by cAMP-PK in isolated membranes from whole glands. Nor do we know why the isoproterenol-stimulated phosphorylated 30,000 M.W. protein was not detected in isolated membranes from whole glands.

The possibility must be considered that the phosphorylation of the low molecular weight substrates detected in this study was due to proteolysis of higher molecular weight proteins such as cAMP-PK itself (20,21). A proteolytic fragment of the 55,000 M.W. R subunit of cAMP-PK_{II} (which can be phosphorylated) has a molecular weight of 37,000 (22). Therefore, experiments were conducted with 5 μM benzamidine and 5 μM phenylmethylsulfonyl fluoride in the homogenization buffer to reduce possible influence of endogenous protease activity during the isolation (Fig. 2). The results were essentially the same as those in the absence of protease inhibitors. The presence of a higher concentration (500 μM) of each inhibitor gave similar results (not shown). Nevertheless we cannot yet completely rule out the presence of cAMP-PK_{II} R subunit at 34,000 M.W.

It has been shown for the parotid gland that β-adrenergic drugs stimulate a separate pathway from either α-adrenergic or muscarinic pathways (7). The present study further extends the investigation of α-adrenergic and cholinergic (muscarinic) actions. These agonists failed to enhance protein phosphorylation at concentrationsand time periods at which stimulated-amylase release was obtained. Thus plasma membrane protein phosphorylation is specific to β-adrenergic stimulation.

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