β -Adrenergic stimulation alters oligosaccharide pyrophosphoryl dolichol metabolism in rat parotid acinar cells

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β -Adrenergic stimulation of rat parotid acinar cells markedly increases [3 H]mannose incorporation into *N*-linked glycoproteins [Kousvelari, Grant, Banerjee, Newby & Baum (1984) *Biochem. J.* 222, 17–24]. More than 90% of this protein-bound [3 H]mannose was preferentially incorporated into four secretory glycoproteins. The ratio of [3 H]mannose/[14 C]leucine present in these individual proteins was 1.7–4-fold greater with isoproterenol-treated cells than with untreated controls. In isoproterenol-stimulated cells, [3 H]mannose incorporation into mannosylphosphoryl dolichol and oligosaccharide-*PP*-dolichol was increased 2–3-fold over that observed in unstimulated cells. Similarly, formation of mannosylated oligosaccharide-*PP*-dolichol was increased approx. 4-fold in microsomes prepared from isoproterenol-treated cells. Also, turnover of oligosaccharide-*PP*-dolichol was significantly increased (5-fold) by β -adrenergic stimulation; the half-life for oligosaccharide-*PP*-dolichol decreased from 6 min in control cells to 1.2 min in isoproterenol-stimulated cells. By 15 min after isoproterenol addition to acinar cells, the specific radioactivity of parotid oligosaccharide moieties increased about 3-fold over the value observed in the absence of the agonist. Taken together, these results strongly suggest that elevation of *N*-linked protein glycosylation in rat parotid acinar cells after β -adrenoreceptor stimulation resulted from significant enhancement in (a) the synthesis of mannosylphosphoryl dolichol and oligosaccharide-*PP*-dolichol and (b) the turnover of oligosaccharide-*PP*-dolichol.

INTRODUCTION

The biosynthesis of oligosaccharides and their attachment to protein asparagine residues is a multi-step process which has received considerable attention in the last decade (Parodi & Leloir, 1979; Hanover & Lennarz, 1982). The process for oligosaccharide-*PP*-dolichol assembly as well as the transfer *en bloc* of the oligosaccharide chain to the appropriate protein acceptor site have been intensively studied (Chapman *et al.*, 1979, 1980; Kornfeld & Kornfeld, 1980). In contrast, relatively little information is available concerning mechanism(s) by which cells may regulate the extent of *N*-linked protein glycosylation.

Many aspects of cellular metabolic function are known to be controlled by hormonal (neurotransmitter, peptide, steroid) signals (Kahn, 1976; Hollenberg, 1979). Precedent certainly exists to suggest that extracellular signalling can influence the *N*-linked glycosylation process (DeRosa & Lucas, 1982; Sarkar & Mookerjee, 1984; Franc *et al.*, 1984). Previously, we have suggested that such extracellular signalling by neurotransmitters could influence the *N*-linked protein glycosylation process (Kousvelari *et al.*, 1983a). We observed that β -adrenoreceptor activation, which is known to regulate protein secretion in rat parotid cells, specifically enhanced *N*-linked protein glycosylation by a cyclic AMP-dependent mechanism (Kousvelari *et al.*, 1983a, 1984).

Since the changes in glycosylation measured were

inhibited by low concentrations of tunicamycin, we reasoned that stimulation of the β -adrenergic receptor may have affected the formation and/or turnover of oligosaccharide-*PP*-dolichol. The present study was carried out to evaluate this question.

EXPERIMENTAL PROCEDURES

Materials

Three-month-old male Wistar rats were purchased from Harlan Sprague-Dawley. All animals were allowed water and NIH-Purina laboratory chow *ad libitum*. Collagenase (CLSPA; lot no. S2C481) was purchased from Worthington Biochemical. Bovine testicular hyaluronidase (type I-S), bovine serum albumin (fraction V); (–)-isoproterenol and dolichyl phosphate were from Sigma. Pronase was obtained from Calbiochem. [4,5- 3 H]Leucine (1 Ci/mmol), [2- 3 H]mannose (2–3 Ci/mmol), [U- 14 C]leucine (302 mCi/mmol), [1- 3 H]glucosamine (17.6 Ci/mmol), GDP-[U- 14 C]mannose (203 mCi/mmol), [3 H]acetic anhydride (40 mCi/mmol) and [14 C]methylated protein mixture consisting of myosin, phosphorylase *b*, bovine serum albumin, carbonic anhydrase and lysozyme were purchased from Amersham. Enlightening was obtained from New England Nuclear. Ham's F-12 medium was from the NIH Media Unit. Filtron X was purchased from National Diagnostics and Bio-Gel P-4 (200–400 mesh) was from Bio-Rad. Concanavalin A-agarose was

Abbreviations used: SDS, sodium dodecyl sulphate; Man-*P*-Dol, mannosylphosphoryl dolichol; GlcNAc-*PP*-Dol, *N*-acetylglucosaminylpyrophosphoryl dolichol; (GlcNAc)₂-*PP*-Dol, *NN'*-diacetylchitobiosylpyrophosphoryl dolichol.

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from BRL Inc. and Sephadex G-25 was from Pharmacia. Fractogel TSK HW 40/S was from EM Sciences.

Preparation of parotid cells

Rats were killed and parotid glands were removed, trimmed of fat and connective tissue and minced quickly. Enzymically dispersed cells were prepared essentially as described previously. Unless otherwise stated, tissue from two rats was used in each experiment (Kousvelari *et al.*, 1983a, 1984). After enzyme digestion, cell aggregates were washed three times with 5 ml of Ham's F-12 low glucose (0.56 mM) medium containing 0.022% bovine serum albumin and resuspended in 2 ml of fresh Ham's F-12 medium (low glucose or leucine free) when radiolabelled saccharides or leucine were used respectively.

Incorporation of radiolabelled tracers into proteins

Following preparation, cells were incubated with or without isoproterenol and either [^3H]mannose (25 $\mu\text{Ci/ml}$), [^{14}C]leucine (2 $\mu\text{Ci/ml}$) or [^3H]glucosamine (35 $\mu\text{Ci/ml}$). Incubations were continued for 60 min. Thereafter cells were separated from the medium by centrifugation (15 s at 40 g), medium was saved on ice and the cells were washed three times with 3 ml of phosphate-buffered saline (pH 7.4). Cells were resuspended in 3 ml of phosphate-buffered saline and homogenized with a Brinkman Polytron (setting 5 for 10 s). The incorporation of radiolabelled tracer into protein was determined as 10% (w/v) trichloroacetic-acid-insoluble material collected on Millipore filters (HA, pore size 0.45 μm). Filters were washed three times with trichloroacetic acid and dissolved in 10 ml of Filtron X. Radioactivity was determined by liquid-scintillation spectrometry.

In experiments where radiolabelled proteins were to be analysed by SDS/polyacrylamide-gel electrophoresis (see below), after preparation cells were preincubated in the absence or presence of 100 μg of dolichyl phosphate before addition of radioisotopes and agonists. Dolichyl phosphate enhances [^3H]mannose incorporation into parotid proteins without affecting either protein synthesis or protein secretion (Kousvelari *et al.*, 1983b), and the effects of isoproterenol are comparable with or without this preincubation procedure.

Incorporation of radiolabelled sugars into saccharide lipids in parotid cells

When the effects of isoproterenol (10^{-5} M) on the incorporation of [^3H]mannose into Man-*P*-Dol and oligosaccharide-*PP*-dolichol, or the incorporation of [^3H]glucosamine into oligosaccharide-*PP*-dolichol, were studied, the prepared cells, from the glands of four rats, were resuspended in 10 ml of medium containing [^3H]mannose (25 $\mu\text{Ci/ml}$) or [^3H]glucosamine (50 $\mu\text{Ci/ml}$) with or without agonist. Aliquots of the cell suspension (2 ml) were removed at various times, washed and homogenized as above, then frozen on solid CO_2 for later analysis. For studies measuring turnover of oligosaccharide-*PP*-dolichol, cells were pulsed with [^3H]mannose (50 $\mu\text{Ci/ml}$) for 30 min. Thereafter cells were washed once with Ham's F-12 medium (3 ml) and resuspended in 12 ml of medium containing 20 mM unlabelled mannose with or without 10^{-5} M-isoproterenol. After various times cells were washed, homogenized and frozen on solid CO_2 as above.

Isolation of saccharide-lipids

Frozen cell homogenates were quickly thawed and then centrifuged (100000 g) at 4 °C for 1.5 h in a Beckman type 42.1 fixed angle rotor. After aspiration of supernatant, the pellet (representing about 10% of the cells obtained from the tissue of four rats) was quickly dispersed into 5 ml of chloroform/methanol (2:1, v/v), allowed to stand for 20 min at room temperature, centrifuged (500 g at 10 °C for 15 min) and the chloroform/methanol supernatant was saved. All subsequent extraction steps to isolate the saccharide-lipids and oligosaccharide-*PP*-dolichol, prior to mild acid hydrolysis, were performed according to the procedure of Lucas *et al.* (1975).

Chromatographic procedures

Analysis of the saccharide units released from Man-*P*-Dol, GlcNAc-*PP*-Dol and (GlcNAc) $_2$ -*PP*-Dol by mild acid hydrolysis was carried out by paper chromatography on Whatman 3 MM paper as reported earlier (Lucas *et al.*, 1975; Grant & Lennarz, 1983). Mild acid hydrolysis of these small saccharide-lipids as well as oligosaccharide-lipids recovered in the extract from chloroform/methanol/water (10:10:3, by vol.), was performed as described (Grant & Lennarz, 1983). Gel filtration chromatography of oligosaccharides on a Bio-Gel P-4 column (1.8 cm $\times$ 110 cm, 200–400 mesh) was carried out using 50 mM-ammonium formate, pH 8.0, containing NaN_3 (0.02%, w/v) as the eluant. [^{14}C]Dextran (10 kDa) and [^{14}C]mannose were used as V_0 (68 ml) and V_i (178 ml) markers, respectively, to calibrate the columns.

Determination of oligosaccharide specific radioactivity in polymannose glycoproteins and in the oligosaccharide-*PP*-dolichol pool

The specific radioactivity of parotid oligosaccharide moieties was measured by two separate approaches. Oligosaccharides were obtained from both polymannose glycoproteins and the cellular oligosaccharide-*PP*-dolichol pool. In the former case cells from the glands of four rats were incubated with [^3H]leucine for 30 min, divided into two portions and then re-incubated in the absence or presence of 10^{-5} M-isoproterenol in medium containing [^{14}C]mannose. After 15 min cells were collected, washed and homogenized as above. Aliquots were removed for analysis of DNA and protein (see below) and then frozen on solid CO_2 . At a later time homogenates were thawed, solubilized [in 20 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl, 10 mM- CaCl_2 , 10 mM- MgCl_2 , 0.5% (v/v) aprotinin and 2.0% (w/v) NP-40] and chromatographed on Sephadex G-25 to remove unincorporated radiolabel. Polymannose glycoproteins were obtained by batch incubation with 5 ml of concanavalin A-agarose (orbital shaking, 4 °C, 4 h). Non-polymannose glycoproteins were removed with washing in the above solution modified to include only 0.02% NP-40. Specific elution of adsorbed polymannose glycoproteins was achieved with buffer containing 300 mM- α -methyl mannoside. Samples were assayed for radioactivity, dialysed against phosphate-buffered saline containing 0.02% NP-40 and equal portions of [^3H]leucine radioactivity were taken from control and isoproterenol-treated samples. Samples were concentrated by lyophilization and subjected to exhaustive Pronase digestion. Resulting glycopeptides

were isolated by chromatography on Bio-Gel P-4 and then acetylated with [^3H]acetic anhydride according to the method of Hoffmann *et al.* (1972). Samples were separated from unincorporated radioisotope by washing six times in chloroform/acetic acid (5:1, v/v), and then purified on a Fractogel TSK HW 40/S column (150 ml bed volume) eluted with chloroform/methanol (2:1, v/v). Eluted glycopeptides were assayed for radioactivity.

For the second approach, [^3H]mannose-labelled oligosaccharide was isolated from cellular oligosaccharide-PP-dolichol after mild acid hydrolysis. Oligosaccharides were desalted on a mixed bed ion-exchange column (5 ml each of Dowex AG1-X8 and Dowex 50X8). Eluted material was washed five times with equal volumes of chloroform/methanol (2:1, v/v) and residual organic solvent was removed under N_2 prior to freezing and lyophilization. Oligosaccharides were then reduced to their alcohol form with 0.23 M- NaBH_4 (Takasaki *et al.*, 1982). Samples were acidified (two drops of glacial acetic acid) and re-chromatographed on a mixed bed ion-exchange column. Non-adsorbed material was flash evaporated (eight times) with 20 ml of acidified methanol. The sample was then dissolved in 2 ml of water, re-chromatographed on a mixed bed ion-exchange column and the eluant was lyophilized and desiccated over P_2O_5 . Thereafter samples were acetylated and analysed by h.p.l.c. as described by Wells *et al.* (1982). Polysaccharide mass was estimated from absorbance at 205 nm with acetylated, reduced stachyose as an internal standard. Greater than 98% of the recovered radioactivity was found under a single u.v.-absorbing peak. Sample recovery was also excellent (greater than 93% of injected [^3H]mannose radioactivity). Sample retention times matched those reported by Wells *et al.* (1982).

Preparation and function of parotid acinar cell microsomes

The dispersed cells, from the tissue of ten rats, were incubated at 37 °C in a shaking waterbath in the presence and absence of 10^{-5} M-isoproterenol in Ham's F-12 medium for 1 h. The cells were gassed with O_2/CO_2 (19:1) at 20 min intervals during the incubation. At the end of incubation, the microsomal membranes were prepared following a procedure described for calf brain (Waechter *et al.*, 1976) with little modification (Banerjee *et al.*, 1985). Membranes were frozen in multiple aliquots until used as the microsomal enzyme source. The enzymic formation of saccharide lipids was measured essentially as described earlier (Banerjee *et al.*, 1985).

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis was carried out as described by Laemmli (1970). Electrophoresis was performed on 4–20% polyacrylamide gradient gels. Samples were prepared as described earlier (Kousvelari *et al.*, 1984). Gels were stained overnight in 0.025% Coomassie Blue and destained by diffusion. Destained gels were soaked in Enlightening for about 20 min before drying. Radioactivity on the gels was detected using methods first described by Bonner & Laskey (1974). M_r values were determined by comparing electrophoretic migration of [^3H]mannose- or [^{14}C]leucine-labelled proteins with that of standard proteins.

Fig. 1. Effect of isoproterenol on the glycosylation of individual parotid proteins

Cells were prepared and incubated with either [^{14}C]leucine or [^3H]mannose in the absence or presence of isoproterenol. After 60 min, medium and cells were separated by centrifugation. Cells were washed, homogenized, and centrifuged at 10000 g for 30 min at 4 °C. Comparable (by amount of DNA) aliquots of the medium and soluble cell fractions were prepared for SDS/polyacrylamide-gel electrophoresis as described under 'Experimental procedures'. After electrophoresis, autoradiograms were prepared and results were then subjected to densitometric analysis. The Figure represents a densitometric scan of medium (secreted) proteins from cells incubated with isoproterenol in the presence of [^3H]mannose. The four glycoproteins seen (M_r values 17000, 32000, 38000 and 220000) are associated with 90% of the protein-bound [^3H]mannose observed (i.e. medium + cellular). Two other major secretory proteins observed when cells are labelled with leucine, but barely detectable when mannose is employed as a tracer, are indicated at M_r values 24000 and 60000 (amylase). Above each of the four glycoprotein peaks, the increase (fold) in glycosylation is indicated. This value was obtained in the following manner. All samples were subjected to electrophoresis simultaneously. Four (medium and cell; [^{14}C]leucine and [^3H]mannose) autoradiograms each were obtained for control and isoproterenol-stimulated cells and scanned at the same time under the same conditions. The relative area of each peak was determined by cutting out, and weighing, the tracings and the values obtained for media and cell combined for each radioisotope. The ratio of total [^3H]mannose area/[^{14}C]leucine area was next calculated. Finally the difference in this ratio between isoproterenol-treated and control cells was determined as fold increase (in parentheses in the Figure). Thus for glycoproteins of M_r approx. 32000 and 38000, isoproterenol results in about a 1.7-fold (70%) increase in the [^3H]mannose/[^{14}C]leucine ratio compared with control incubations. Similarly, for the M_r 17000 glycoprotein this increase is about 2.5-fold (150%), while glycosylation of the M_r 220000 protein is enhanced about 4-fold (300%).

Quantification of the radioactivity associated with each protein band was accomplished by densitometric scanning of autoradiograms using a GS300 Scanning Densitometer (Hoefer Scientific) attached to an Omniscribe D-5000 strip chart recorder.

Miscellaneous procedures

The DNA content of cellular homogenates was determined by the diphenylamine method as described by Richards (1974). Protein was assayed by the method of Lowry *et al.* (1951).

Fig. 2. Effect of isoproterenol on the incorporation of $[^3\text{H}]$ mannose into oligosaccharide-PP-dolichol and Man-P-Dol

Cells (5.0 mg of protein/ml) were incubated in the absence (●) or presence (▲) of isoproterenol (10^{-5} M) in medium containing $[^3\text{H}]$ mannose (25 $\mu\text{Ci}/\text{ml}$). Aliquots (2.0 ml) were taken at the indicated times and cells obtained by centrifugation (100 g for 15 s). The cell pellet was resuspended and homogenized as described under 'Experimental procedures'. $[^3\text{H}]$ Mannose (reported as per mg of protein) incorporation into (a) oligosaccharide-PP-dolichol and (b) Man-P-Dol was measured. Data shown represent the average of two separate determinations. The standard deviations about each point varied from 5 to 15% of the mean values.

RESULTS

Demonstration of $[^3\text{H}]$ saccharide incorporation into parotid glycoproteins

Rat parotid acinar cells respond to β -adrenoreceptor stimulation by increasing $[^3\text{H}]$ mannose incorporation into N -linked glycoproteins (a 2-fold increase in $[^3\text{H}]$ mannose/ $[^{14}\text{C}]$ leucine ratio, 0.22 to 0.45; similar to results previously reported by Kousvelari *et al.*, 1984). In the same cell preparations, isoproterenol also caused a modest but consistent increase in $[^3\text{H}]$ glucosamine incorporation into N -linked glycoproteins (a 33% increase in $[^3\text{H}]$ glucosamine/ $[^{14}\text{C}]$ leucine ratio, 0.115 to 0.153).

The endogenous parotid protein acceptors, accounting for the increased $[^3\text{H}]$ mannose incorporation, were examined by subjecting secreted and cellular proteins to SDS/polyacrylamide-gel electrophoresis and autoradiography. In the presence of isoproterenol, most $[^3\text{H}]$ mannose (> 90%) is incorporated into four secretory glycoproteins (Fig. 1) despite the fact that parotid acinar cells synthesize many different protein species *in vitro* (Kousvelari *et al.*, 1983b, 1984). Glycosylation of these proteins is seen in control incubations but at much lower levels. Similarly, little release of secretory proteins occurs without β -adrenergic stimulation (Schramm & Selinger, 1975). In order to assess the extent of glycosylation observed, we subjected autoradiograms of parotid proteins radiolabelled with either $[^{14}\text{C}]$ leucine or

$[^3\text{H}]$ mannose to a densitometric evaluation procedure and determined the ratio of radioactivity incorporated into each of the four glycoproteins of interest (cellular plus secreted forms). As shown in Fig. 1, two secretory glycoproteins (M_r approx. 32000 and 38000) show about a 1.7 fold-elevation in glycosylation ($[^3\text{H}]$ mannose/ $[^{14}\text{C}]$ leucine), while a third (M_r approx. 17000) is enhanced 2.5-fold compared with control gels. In the fourth secretory glycoprotein (M_r approx. 220000) this ratio is increased by about 4-fold. These results agree quite well with values obtained by examining similar ratios of trichloroacetic-acid-insoluble parotid protein (e.g. Kousvelari *et al.*, 1983a, 1984) and strongly support the notion that the β -adrenergic agonist specifically increases the mannosylation of parotid proteins concomitant with eliciting protein secretion.

Although not depicted in Fig. 1, ratio data was obtained on these four glycoproteins in separate cellular and secreted forms. Cellular glycoproteins from control preparations displayed $[^3\text{H}]$ mannose/ $[^{14}\text{C}]$ leucine ratios about 50% lower than obtained from isoproterenol-treated preparations (41%, 53%, 50% and 45% for proteins of M_r 17000, 32000, 38000 and 22000 respectively), while secreted glycoproteins from control cells showed a markedly lower ratio than that of isoproterenol-treated cells (9%, 3%, 1% and 0.4% for proteins of M_r 17000, 32000, 38000 and 220000 respectively). Thus glycoproteins secreted under β -adrenoreceptor stimulation show the highest degree of $[^3\text{H}]$ mannosylation.

Fig. 3. Effect of isoproterenol on the incorporation of $[^3\text{H}]$ glucosamine into oligosaccharide-PP-dolichol

Cells were incubated in the absence (●) or presence (▲) of isoproterenol (10^{-5} M) in medium containing $[^3\text{H}]$ glucosamine (50 $\mu\text{Ci}/\text{ml}$). Isolation of oligosaccharide-PP-dolichol and determination of radioactivity incorporated (reported as per mg of protein) were similar to that described in Fig. 2 and under 'Experimental procedures'. Data shown represent the average of two separate determinations. The standard deviations about each point varied from 10 to 20% of the mean values.

Effect of β -adrenergic stimulation on oligosaccharide-PP-dolichol synthesis by parotid cells

When parotid acinar cells were incubated *in vitro* with $[^3\text{H}]$ mannose in the presence of isoproterenol a 3.5-fold increase in $[^3\text{H}]$ mannose incorporation into oligosaccharide-PP-dolichol (chloroform/methanol/water, 10:10:3, by vol.) was seen as early as 2.5 min after receptor activation (Fig. 2a). The rate of $[^3\text{H}]$ mannose incorporation decreased thereafter for both control and hormone-treated cells with apparent saturation after 10–30 min. Similarly, $[^3\text{H}]$ mannose incorporation into Man-P-Dol (chloroform/methanol, 2:1, v/v) was also markedly (approx. 2-fold) increased in isoproterenol-

treated acinar cells when compared with control cells at early time points (Fig. 2b). Rate of $[^3\text{H}]$ mannose incorporation decreased somewhat after about 10 min in agonist-treated cells, whereas basal formation of $[^3\text{H}]$ Man-P-Dol continued to be reasonably proportional to time over the experimental period.

In the presence of isoproterenol, an increase in $[^3\text{H}]$ mannose incorporation into oligosaccharide-PP-dolichol might reflect changes either in synthesis *de novo* of the lipid-linked oligosaccharide and/or in oligosaccharide chain elongation. As an approach to examine this question and attempt to estimate oligosaccharide-lipid synthesis *de novo*, we evaluated the effect of isoproterenol on $[^3\text{H}]$ glucosamine incorporation into the oligosaccharide-PP-dolichol fraction. As shown in Fig. 3, β -adrenergic receptor stimulation initially resulted in little or no increase in $[^3\text{H}]$ glucosamine incorporation into oligosaccharide-PP-dolichol. However, at later time points (16–24 min) significant elevations were seen (approx. 1.35-fold). This is comparable with the increase in $[^3\text{H}]$ glucosamine incorporation into N-linked glycoproteins described above. Also similar results were observed for GlcNAc-PP-dolichol and (GlcNAc)₂-PP-dolichol isolated from the chloroform/methanol (2:1, v/v) extract of parotid cells (results not shown).

Saccharide-lipid formation by parotid acinar cell microsomes *in vitro*

Previously we have shown that, after treatment with isoproterenol, microsomes from parotid acinar cells show increased saccharide-lipid formation *in vitro* (Banerjee *et al.*, 1985). To be sure that enhanced incorporation of $[^3\text{H}]$ mannose into oligosaccharide-PP-dolichol (Fig. 2a) was due only to transfer of mannose from Man-P-Dol, and not due to a direct transfer from GDP-mannose, we examined the effect of amphomycin on saccharide-lipid formation by parotid microsomes. As shown in Table 1 (similar to data reported by Banerjee *et al.*, 1985), microsomes from isoproterenol-treated cells show about a 33% increase in Man-P-Dol formation *in vitro* when assayed in the presence of Mn^{2+} . Formation of $[^3\text{H}]$ mannosylated oligosaccharide-PP-Dol is increased about 4-fold. Incubation of microsomes in the presence of Ca^{2+} prevents optimum saccharide-lipid formation but is necessary for amphomycin action (Banerjee *et al.*, 1981). As can be seen in Table 1, amphomycin clearly

Table 1. Formation of mannosylated saccharide-lipids in microsomal membranes in the presence and absence of amphomycin

Synthesis of $[^{14}\text{C}]$ mannose-labelled saccharide-lipids was assayed by incubating microsomal membranes prepared from control or isoproterenol-treated parotid acinar cells in 5 mM-Tris/HCl, pH 7.0, containing 12.5 mM-sucrose, 50 μM -EDTA, 2.5 μM -GDP- $[^{14}\text{C}]$ mannose (593 c.p.m./pmol) and either MnCl_2 or 5 μM - CaCl_2 in the presence or absence of amphomycin at 37 °C for 5 min. Saccharide lipids were assayed as described under 'Experimental procedures'.

Additions	Man-P-Dol (c.p.m./mg of protein)	Mannosylated oligosaccharide-PP-Dol (c.p.m./mg of protein)
Untreated control (Mn^{2+})	20816	440
Isoproterenol-treated (Mn^{2+})	27774	1964
Untreated control (Ca^{2+})	4560	694
Untreated control (Ca^{2+}) + amphomycin (5 μg)	316	36
Isoproterenol-treated (Ca^{2+})	4636	3272
Isoproterenol-treated (Ca^{2+}) + amphomycin (5 μg)	1702	658

Table 2. Effects of isoproterenol on oligosaccharide specific radioactivity

Oligosaccharide specific radioactivity was measured by two separate approaches. In Expt. 1 oligosaccharides were obtained from polymannose glycoproteins. Data are the results of a single experiment performed as described under 'Experimental procedures'. In Expt. 2 oligosaccharides were obtained from the cellular oligosaccharide-*PP*-dolichol pool. Data shown are the means $\pm$ S.E.M. for three separate determinations as described under 'Experimental procedures'.

Expt.	Treatment	[^{14}C]Mannose in oligosaccharides (c.p.m.)	^3H in acetylated oligosaccharides (c.p.m.)	Ratio $^{14}\text{C}/^3\text{H}$ (specific radioactivity)	Increase above control (fold)
1	Control	33 560	7840	4.28	—
	Isoproterenol	50 000	3811	13.12	3.06
		[^3H]Mannose in oligosaccharides (c.p.m.)	Total mass of oligosaccharide/mg of protein (nmol)	Specific radioactivity (c.p.m./mol)	
2	Control	20 310 $\pm$ 1864	11.3 $\pm$ 1.66	1.78 $\times 10^{12}$	—
	Isoproterenol	47 290 $\pm$ 3224	8.47 $\pm$ 0.66	5.58 $\times 10^{12}$	3.13

Fig. 4. Analysis of oligosaccharide size by chromatography on Bio-Gel P-4

Oligosaccharides were obtained from the cellular oligosaccharide-*PP*-dolichol pool of control and isoproterenol-treated cells, and gel filtration chromatography was performed, as described under 'Experimental procedures'. $\circ$, Control; $\bullet$, isoproterenol-treated. V_0 was 68 ml, V_i was 178 ml.

inhibits formation of Man-*P*-Dol and oligosaccharide-*PP*-Dol by microsomes from both control and isoproterenol-treated cells.

Effect of isoproterenol on oligosaccharide specific radioactivity and on the turnover of oligosaccharide-*PP*-dolichol

The specific radioactivity (c.p.m. of mannose radio-label/unit mass) of oligosaccharides obtained from polymannose glycoproteins and from the cellular oligosaccharide-*PP*-dolichol pool was increased about 3-fold in isoproterenol-treated cells compared with controls (Table 2). In each case this effect was due in part to higher incorporation of radiolabelled mannose into oligosaccharides after isoproterenol treatment. In addition the apparent mass of oligosaccharide, either as per unit

polypeptide core when polymannose glycoprotein oligosaccharides were studied or per mg cellular protein when the oligosaccharide-*PP*-dolichol pool was evaluated, was significantly lower in agonist-treated cells. This decrease in oligosaccharide mass was not due to a reduction in mannose residues per oligosaccharide chain; rather it resulted from a reduction in total mass of apparently fully sized oligosaccharide chains (Fig. 4).

These results suggested the possibility that the oligosaccharide-*PP*-dolichol pool might turn over at a much higher rate in the presence of isoproterenol. As shown in Fig. 5, it was apparent that isoproterenol treatment resulted in significantly increased turnover of this pool. In control cells [^3H]mannose-labelled oligosaccharide-*PP*-dolichol had a half-life of 6 min compared with a half-life of 1.2 min seen with isoproterenol-stimulated cells.

DISCUSSION

There exists considerable evidence demonstrating that activation of cell surface hormone receptors regulates a wide variety of cellular metabolic events including protein production and processing. Many extracellular signals have been shown to influence both protein biosynthesis (e.g. Korc, 1982; Hobbs *et al.*, 1982; Firestone *et al.*, 1982) and intracellular protein turnover (Bienkowski, 1983). However, less is known about the effect of hormone stimulation on covalent post-translational modifications of proteins, in particular *N*-linked oligosaccharide addition and processing (e.g. DeRosa & Lucas, 1982; Sarkar & Mookerjee, 1984; Franc *et al.*, 1984). We have reported recently that β -adrenoreceptor activation was capable of enhancing *N*-linked protein glycosylation by a cyclic AMP-mediated process in rat parotid acinar cells (Kousvelari *et al.*, 1983a, 1984). It was the purpose of the present series of experiments to examine several possible mechanistic explanations for this phenomena, in particular alterations in oligosaccharide-*PP*-dolichol metabolism.

The results presented in Fig. 1 demonstrate that isoproterenol markedly increases glycosylation of four secretory glycoproteins. Although acute β -adrenoreceptor

Fig. 5. Effect of isoproterenol on the turnover of [3 H]mannose-labelled oligosaccharide-PP-dolichol

Cells were preincubated in the presence of [3 H]mannose (50 μ Ci/ml) for 30 min (a time adequate to label maximally the oligosaccharide-PP-dolichol pool; Fig. 2a). Cells were washed, resuspended in medium containing 20 mM unlabelled mannose in the absence ($\bullet$) or presence ($\blacktriangle$) of isoproterenol (10^{-5} M). Aliquots (2.0 ml) were taken at indicated times. Extraction and determination of oligosaccharide-PP-dolichol (reported as per mg of protein) were identical with that described in Fig. 2 and under 'Experimental procedures'. Data shown are the average of two separate determinations performed with cells from pooled glands of four rats. Variation between duplicates was about 5–15% of mean values. Through the 5 min time point there was no overlap in data points. The average ($\pm$ S.E.M.) for control values at 1.5, 2.5 and 5 min was 400 ± 34 c.p.m./mg of protein (i.e. six data points) while for isoproterenol-treated cells this value was 238 ± 14 .

stimulation of parotid cells *in vitro* may result in increased protein synthesis (Kousvelari *et al.*, 1983a, 1984), this change is small compared with changes in protein glycosylation. For each of the four secretory glycoproteins the overall degree of [3 H]mannosylation is significantly greater than the protein labelling with [14 C]leucine. We also noted that glycosylation appeared particularly enhanced in the secreted forms (in the medium) of these glycoproteins. It has been proposed that oligosaccharide moieties may be particularly important in maintaining protein solubility and conformation (Olden *et al.*, 1985). β -Adrenoreceptor stimulation results in copious exocrine protein release into the fairly confined space of a duct lumen. It is tempting to speculate that this event which signals protein secretion also signals a protein modification step which may promote exocrine protein solubility, and thus delivery to their site of function.

Essentially all other data presented here (Figs. 2, 3 and 5 and Tables 1 and 2) are consistent with a direct influence of the β -adrenergic receptor on the lipid-linked oligosaccharide pathway resulting in elevated *N*-linked protein glycosylation. These findings suggest both the increased formation of, and the increased turnover of (addition to acceptor), oligosaccharide-PP-dolichol as early responses to isoproterenol treatment. These events would explain increased radiolabelling of the oligosaccharide-PP-

dolichol pool and result in the need for more, and temporally later, formation *de novo* of oligosaccharide-PP-dolichol (i.e. estimated here as [3 H]glucosamine labelled oligosaccharide lipid). Furthermore, experiments by us (S. R. Grant, J. K. Welply, E. E. Kousvelari & B. J. Baum, unpublished work) have demonstrated a significant increase in protein-oligosaccharide transferase activity found in microsomal membranes from isoproterenol-treated cells.

Thus, in aggregate, the results of the present investigation provide strong support for β -adrenoreceptor regulation of oligosaccharide-PP-dolichol metabolism during *N*-linked protein glycosylation. The rat parotid cell provides a useful, well-characterized (with respect to neurotransmitter-signal transduction and exocrine physiology) model to examine hormonal control of these glycosylation events. Clearly, further detailed study of such control is warranted.

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REFERENCES

- Banerjee, D. K., Scher, M. G. & Waechter, C. J. (1981) *Biochemistry* **20**, 1561–1568
- Banerjee, D. K., Kousvelari, E. E. & Baum, B. J. (1985) *Biochem. Biophys. Res. Commun.* **126**, 123–129
- Baum, B. J., Ito, H. & Roth, G. S. (1983) in *Adrenoreceptors and Catecholamine Action, Part B* (Kunos, G., ed.), pp. 265–294, Wiley, New York
- Bienkowski, R. S. (1983) *Biochem. J.* **214**, 1–10
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Butcher, F. R. & Putney, J. W., Jr. (1980) *Adv. Cyclic Nucleotide Res.* **13**, 215–249
- Chapman, A., Trowbridge, I. S., Hyman, R. & Kornfeld, S. (1979) *Cell* **17**, 509–515
- Chapman, A., Fujimoto, R. & Kornfeld, S. (1980) *J. Biol. Chem.* **255**, 4441–4446
- DeRosa, P. A. & Lucas, J. J. (1982) *J. Biol. Chem.* **257**, 1017–1024
- Firestone, G. L., Payvar F. & Yamamoto, K. R. (1982) *Nature (London)* **300**, 221–225
- Franc, J.-L., Hovsepian, S., Fayet, G. & Bouchilloux, S. (1984) *Biochem. Biophys. Res. Commun.* **118**, 910–915
- Grant, S. R. & Lennarz, W. J. (1983) *Eur. J. Biochem.* **134**, 575–583
- Hanover, J. A. & Lennarz, W. J. (1982) *J. Biol. Chem.* **257**, 2787–2794
- Hobbs, A. A., Richards, D. A., Kessler, D. J. & Rosen, J. M. (1982) *J. Biol. Chem.* **257**, 3598–3605
- Hoffmann, J., Lindberg, B. & Svensson, S. (1972) *Acta Chem. Scand.* **26**, 661–666
- Hollenberg, M. D. (1979) *Pharmacol. Rev.* **30**, 393–410
- Kahn, C. R. (1976) *J. Cell Biol.* **70**, 261–286
- Korc, M. (1982) *Am. J. Physiol.* **243**, 69–75
- Kornfeld, R. & Kornfeld, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 1–36, Plenum Press, New York
- Kousvelari, E. E., Grant, S. R. & Baum, B. J. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7146–7150
- Kousvelari, E. E., Grant, S. R. & Baum, B. J. (1983b) *Exp. Cell Res.* **149**, 271–276
- Kousvelari, E. E., Grant, S. R., Banerjee, D. K., Newby, M. J. & Baum, B. J. (1984) *Biochem. J.* **222**, 17–24
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lucas, J. J., Waechter, C. J. & Lennarz, W. J. (1975) *J. Biol. Chem.* **250**, 1992–2002
- Olden, K., Bernard, B. A., Humphries, M. J., Yeo, T.-K., Yeo, K.-T., White, S. L., Newton, S. A., Bauer, H. C. & Parent, J. B. (1985) *Trends Biochem. Sci.* **10**, 78–82
- Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
- Richards, G. M. (1974) *Anal. Biochem.* **57**, 369–376
- Sarkar & Mookerjee, S. (1984) *Biochem. J.* **219**, 429–436
- Schramm, M. & Selinger, Z. (1975) *Methods Enzymol.* **39**, 461–466
- Takasaki, S., Mizuochi, T. & Kobata, A. (1982) *Methods Enzymol.* **83**, 263–268
- Waechter, C. J., Kennedy, K. L. & Harford, J. B. (1976) *Arch. Biochem. Biophys.* **174**, 726–737
- Wells, G. B., Kontoyiannidou, V., Turco, S. J. & Lester, R. L. (1982) *Methods Enzymol.* **83**, 132–137

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