Extensive destruction of newly synthesized casein in mammary explants in organ culture

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(Received 27 July 1981/Accepted 9 September 1981)

1. Explants of mammary glands of mid-pregnant rabbits that had been cultured for 18 h in the presence of insulin, prolactin and cortisol were incubated at 37°C for 2 h in Medium 199 containing L-[4,5-³H]leucine. After a wash procedure at 4°C, explants were re-incubated at 37°C in fresh medium and the radioactivity of casein polypeptides isolated by isoelectric focusing (at pH 4.6) was followed with time. Casein radioactivity rose during the first hour of re-incubation, but fell markedly during the subsequent hour. 2. Loss of radioactivity represented casein degradation, since less than 10% of newly synthesized casein was found in the incubation medium. 3. Such a loss of radioactivity was not due solely to hydrolysis of signal peptides, since similar results were obtained when L-[5-³H]proline, which is not part of casein signal peptides, was the radiolabelled precursor. 4. A dual-isotope experiment using L-[U-¹⁴C]proline and N-[³H]acetyl-D-mannosamine gave similar profiles of radioactivity loss from isoelectrically focused casein, indicating that degradation of mature casein was occurring. 5. Analysis of total pellet and particle-free-supernatant fractions prepared by centrifugation of explant homogenates at 115 000 *g*_{av}. for 1 h did not show loss of radioactivity on re-incubation. Total pellet-protein radioactivity remained constant, whereas total soluble-protein radioactivity increased during the 2 h re-incubation period. 6. Radioactivity in a specific particle-free-supernatant polypeptide, the subunit of fatty acid synthetase, mimicked that of the total soluble protein. 7. Addition of cycloheximide (20 µg/ml) during the re-incubation period completely blocked the incorporation of radioactivity from L-[5-³H]proline into casein and the subsequent fall, indicating that observations were being made on newly synthesized casein. 8. Addition of chloroquine (50 µM) did not prevent the increase in radioactivity from L-[5-³H]proline into casein during the first hour of re-incubation, but did prevent the loss of radioactivity in the second hour. 9. The intracellular degradation of a newly synthesized milk protein is discussed in relation to the known intracellular degradation of other secretory polypeptides.

Many proteins and polypeptides destined for export from the cell undergo extensive degradation during their passage from the smooth endoplasmic reticulum to the plasma membrane (e.g., prolactin, parathyrin, collagen, insulin). Thus, for example up to 70% of newly synthesized parathyrin may be destroyed intracellularly rather than being secreted from the cell (Bienkowski *et al.*, 1978; Shenai & Wallis, 1979; Morrissey & Cohn, 1979; Halban & Wollheim, 1980).

Little is known of the fate of newly synthesized casein during its packaging into secretory vesicles in the Golgi and its transport to the plasma membrane

and into the alveolar lumen. This major secretory protein of the lactating mammary gland is synthesized on membrane-bound polyribosomes (Houdebine & Gaye, 1975; Harrison *et al.*, 1976) with a signal peptide that undergoes co-translational proteolytic cleavage (Craig *et al.*, 1979; Mercier & Gaye, 1980). Little or no degradation of radio-labelled casein was seen in mammary explants cultured in the presence of hormones when measured 24 and 48 h after an initial 1 h pulse of radioactivity (Wilde *et al.*, 1980). Removal of hormones from the tissue resulted in considerable degradation, presumably owing to an autophagic process. However, if

degradative processes, such as those described above as being active during the synthesis of parathyrin, are also active during casein synthesis, measurements of degradation of newly synthesized casein must be made over much shorter time periods. The present paper describes such measurements.

Materials and methods

Animals

Mid-pregnant rabbits (day 16 of pregnancy) were obtained from the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. The animals were at least 6 months old at the time of mating.

Materials

L-[4,5-³H]-Leucine, L-[5-³H]proline, L-[U-¹⁴C]-proline and *N*-[³H]acetyl-D-mannosamine were purchased from Amersham International, Little Chalfont, Bucks., U.K. Ampholytes (pH range 3–10) were from Pharmacia (G.B.), Hounslow, Middx., U.K., and ampholytes (pH range 5–7) were from Bio-Rad Laboratories, Watford, Herts., U.K. Ox insulin was a gift from Mr. J. V. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K. Cortisol 21-acetate, nystatin, benzylpenicillin and streptomycin sulphate were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Sheep prolactin (NIAMDD-OPRL-14; 31.0 i.u./mg) was generously given by Dr. Salvatore Raiti, Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism and Digestive Diseases, National Pituitary Agency, Baltimore, MD, U.S.A. Medium 199 (Morgan *et al.*, 1950) was purchased from Wellcome Research Laboratories, Beckenham, Kent, U.K. All other chemicals and solvents were of A.R. grade and obtained from commercial suppliers.

Preparation and culture of mammary explants

Explants of lobuloalveolar mammary tissue were dissected from mid-pregnant rabbits (day 16 of pregnancy) as described by Forsyth & Myers (1971), and cultured for 18 h at 37°C in Medium 199 in the presence of insulin (5 µg/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml) as described by Al-Sarraj *et al.* (1979).

Incubation of explants

After 18 h in culture, explants were incubated for 2 h at 37°C in 5 ml of Medium 199 containing L-[4,5-³H]leucine (2.4 mCi, sp. radioactivity 72 Ci/mmol), L-[5-³H]proline (2.5 mCi, sp. radioactivity 37 Ci/mol), L-[U-¹⁴C]proline (250 µCi, sp. radioactivity 285 Ci/mol) or *N*-[³H]acetyl-D-mannosamine (1 mCi, sp. radioactivity 500 Ci/mol). The

explants were then washed with ice-cold Medium 199 (4 × 5 ml) containing the appropriate non-radio-labelled precursor at a concentration of 10 mM. Groups of explants were then incubated in Medium 199 at 37°C for 30, 60, 90, 120 and 180 min. When the effects of chloroquine or cycloheximide were studied, the drugs were added to the incubation medium immediately after the wash procedure.

Preparation of tissue extracts

At the end of the incubation the explants were homogenized at 4°C by ten strokes in a glass/glass homogenizer, in 10 mM-Tris/HCl, pH 7.0, containing 0.25 M-sucrose, 1 mM-dithiothreitol and 2 mM-phenyl-methanesulphonyl fluoride. Particle-free supernatant and pellet fractions were obtained by centrifugation of the homogenate at 115 000 g for 1 h at 4°C. Pellet fractions were resuspended in 20 mM-Tris/HCl, pH 7, containing 8 M-urea, 5% (v/v) Triton X-100, 1% (v/v) 2-mercaptoethanol, 0.5% sodium dodecyl sulphate and 1.6 mM-EDTA for analysis by electrophoresis.

Measurements of incorporation of radiolabelled [5-³H]proline precursors into particle-free supernatant and pellet proteins

The incorporation of radiolabelled proline into trichloroacetic acid-insoluble proteins of both particle-free supernatant and resuspended pellet fractions was measured as described by Speake *et al.* (1975). Bovine serum albumin (1 mg) was added to the particle-free supernatant and resuspended pellet fractions, and proteins were precipitated by addition of trichloroacetic acid (final concn. 5%, w/v). Precipitates were collected by centrifugation at 1400 g for 6 min at ambient temperature and washed with trichloroacetic acid (5%, w/v; 3 × 1 ml) containing 10 mM unlabelled substrate (precursor). The washed pellets were dissolved in formic acid (90%, w/v; 0.2 ml) and assayed for radioactivity in a liquid-scintillation counter.

Measurements of the incorporation of L-[5-³H]-proline into subunits of fatty acid synthetase

Portions of the supernatant fraction obtained from incubation of explants with L-[5-³H]proline were analysed by polyacrylamide-gel electrophoresis by the method of Laemmli (1970), but without a stacking gel. The acrylamide concentration was 5%. Fatty acid synthetase (10 µg) was added as carrier to each sample before electrophoresis. Gels were stained with Coomassie Blue, and bands corresponding to the subunits of fatty acid synthetase were cut from the gel and assayed for radioactivity as described by Paskin & Mayer (1978).

Measurement of the incorporation of L-[4,5-³H]-leucine and L-[5-³H]proline into casein

Resuspended pellet fractions of explants that had been incubated in the presence of L-[4,5-³H]leucine or L-[5-³H]proline were subject to isoelectric focusing as described by O'Farrel (1975) after addition of rabbit casein (50 µg) as carrier. Tube gels contained 2% (w/v) Ampholines (1.2%, w/v, pH range 3–5; 0.8%, w/v, pH range 3–10).

Results

Preliminary experiments performed to determine the distribution of newly synthesized casein after fractionation of mammary explants indicated that much (78%) remained in a membrane pellet on homogenization. Presumably this casein is contained in secretory vesicles. The routine isolation and assay of radioactivity incorporated into casein by immunoadsorption on Sepharose–anti-casein columns and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Al-Sarraj *et al.*, 1978a) was laborious and time-consuming. Preliminary experiments using isoelectric focusing indicated that all the radioactivity that focused at pH 4.6 could be bound to the

Sepharose–anti-casein immunoabsorbent columns, and thus isoelectric focusing was used as a simple rapid method of isolating casein polypeptides. All subsequent experiments that involved measurements of radioactivity incorporated into or remaining in casein used this technique. The polypeptides focusing at pH 4.6 were sliced from the gel and processed for radioactivity assay as described above.

Measurement of radioactivity in total protein of pellet and particle-free supernatant fractions

When explants that had been prelabelled by incubation in the presence of L-[5-³H]proline were washed and re-incubated in medium containing no radioactive substrate, the total radioactivity of pellet proteins remained constant over an incubation period of 2 h (Fig. 1a). During this time incorporation of radioactivity into total proteins of the particle-free supernatant fraction and also into the subunit of fatty acid synthetase increased steadily (Fig. 1b).

When similar measurements were made on casein polypeptides isolated by isoelectric focusing, a very different situation obtained. Radioactivity from leucine continued to be incorporated into casein for

Fig. 1. Incorporation of L-[5-³H]proline into (a) trichloroacetic acid-precipitable protein of membrane pellet and (b) particle-free supernatant fraction and fatty acid synthetase

Explants were cultured with hormones for 18 h and then incubated with L-[5-³H]proline for 2 h at 37°C under O₂/CO₂ (19:1) in Medium 199 containing hormones. The explants were then washed four times with Medium 199 (5 ml) containing hormones and proline (10 mM). Groups of 40 explants were chased for the indicated periods. At each time point the explants were weighed and homogenized, and particle-free supernatant and pellet fractions were prepared as described in the Materials and methods section. Results are expressed as means $\pm$ half the difference of two observations. Radioactivity was measured in trichloroacetic acid-precipitable protein from resuspended pellets (●, a), particle-free supernatant fractions (●, b) and also the subunit of fatty acid synthetase (▲).

between 60 and 90 min after the pulse-and-wash procedure, being at least doubled over this period. Subsequently, radioactivity in these polypeptides was lost such that the radioactivity remaining after 180 min was 90% of that at zero time and only 37% of the apparent maximal value at 60 min. This rapid loss of 63% of radioactivity from casein was not prolonged after 180 min of re-incubation, and losses over a further 28 h period were very small (Fig. 2). Similar results were seen when L-[5-³H]proline was used as precursor. Again an increased incorporation of radioactivity into casein polypeptides was apparent during the first hour of re-incubation, followed by a 55% loss of radioactivity over the second hour. A dual-isotope experiment in which explants were incubated with L-[U-¹⁴C]proline and N-[³H]acetyl-D-mannosamine was performed to compare the fates of radioactivity in the peptide backbone and polysaccharide chains (Fig. 3). Incorporation of both radioisotopes into casein polypeptides increased for approx. 60 min. There followed a loss of radioactivity from both substrates (50% of ¹⁴C and 35% of 33% of ³H) over the second hour of incubation, with no further loss during the third hour.

Fig. 2. Incorporation of L-[4,5-³H]leucine into casein. After culture with hormones for 18 h, explants were incubated with L-[4,5-³H]leucine for 2 h at 37°C under O₂/CO₂ (19:1). Explants were washed and re-incubated in groups of 40 for the times indicated. The pellet fraction was obtained at each time point as described in the Materials and methods section. Results are expressed as means ± half the difference between two measurements.

Fig. 3. Incorporation of L-[U-¹⁴C]proline and N-[³H]acetyl-D-mannosamine into casein.

Explants were cultured and washed as described in the legend to Fig. 1. Washed explants were incubated in Medium 199 (5 ml) in the presence of L-[U-¹⁴C]proline (50 µCi/ml) and N-[³H]acetyl-D-mannosamine (200 µCi/ml) for 2 h at 37°C. The pellet fraction was obtained at each time point as described in the Materials and methods section. Incorporation of radioactivity into isoelectrically focused casein was measured as described in the Materials and methods section. Results are means ± half the difference between two observations calculated as a percentage of the maximum radioactivity in casein (i.e. at $t = 60$ min).

Effects of chloroquine and cycloheximide on loss of radioactivity from casein polypeptides

The drugs were added immediately after the washing procedure. The radioactivity from L-[5-³H]proline incorporated into casein in the presence of chloroquine was not significantly different from control values during the first hour of re-incubation (Fig. 4). However, whereas the radioactivity in casein from control explants fell markedly on further incubation, no loss of radioactivity was seen in the casein isolated from explants re-incubated in the presence of chloroquine. Inclusion of cycloheximide in the re-incubation medium prevented the incorporation of radiolabel into casein seen in the first hour in the control explants, and no loss of radioactivity was seen subsequently.

Discussion

The experiments reported here describe the fate of newly synthesized casein, i.e. that synthesized during a 2 h pulse incubation with radiolabelled precursor, in the membrane-pellet fraction. The initial increase in radioactivity of casein polypeptides is probably due to utilization of a highly radioactive intra-

Fig. 4. Effect of chloroquine (O) and cycloheximide (▲) on the synthesis and degradation of newly synthesized casein

Cultured explants were incubated with L-[5-³H]-proline (500 μ Ci/ml) for 2 h and washed as described in the Materials and methods section. Groups of 40 explants were re-incubated in Medium 199 containing hormones (●) and chloroquine (50 μ M) or cycloheximide (20 μ g/ml). The pellet fraction was obtained at each time point as described in the Materials and methods section. The radioactivity in isoelectrically focused casein was measured as described in the Materials and methods section. Results are expressed as means $\pm$ half the difference between two measurements for the cycloheximide experiments and as means $\pm$ S.E.M. for the other experiments. The numbers of experiments are indicated in parentheses.

with unlabelled amino acid will lead to an exponential decline in precursor specific radioactivity and a hyperbolic incorporation of radioactivity into a biosynthetic end-product (Zak *et al.*, 1979). However, this is not true for casein, and total radioactivity in the casein fraction falls rapidly, suggesting a high rate of degradation. Less than 10% of casein radioactivity was found in the incubation medium (results not shown).

It is well established that casein is synthesized, like many other secretory proteins, as a preprotein and that a signal peptide is cleaved as a co-translational event during processing of the proteins (Craig *et al.*, 1979; Mercier & Gaye, 1980). The signal peptide of rabbit β -casein contains 15 amino acid residues, of which five are leucine. Thus much leucine will be lost during cleavage of the signal and might account for some of the loss of radioactivity seen in Fig. 2. To determine whether the loss of radioactivity was due to signal cleavage or general degradation of casein, the pulse experiments were repeated with L-[5-³H]proline as radioactive precursor. This amino acid is a major constituent of the five polypeptides isolated from rabbit casein by Al-Sarraj *et al.* (1978b) and is absent from the signal peptide of β -casein (Mercier & Gaye, 1980). Examination of total pellet proteins, including casein, after a 2 h pulse with [5-³H]proline showed that the radioactivity in this fraction remained constant over the 2 h re-incubation in medium containing no radio-labelled precursor (Fig. 1a). This contrasted with total protein of the soluble fraction, where incorporation of radioactivity increased during the re-incubation period (Fig. 1b). The radioactivity in casein, however, after an initial increase during the first hour fell markedly during the second hour of re-incubation, mimicking the situation when leucine was the radioactive precursor. A fall in the radioactivity (of 55%) in casein suggests that at least this percentage of the newly synthesized casein was degraded during the re-incubation. During this time the radioactivity in a specific soluble protein, fatty acid synthetase, rose in a similar fashion to the radioactivity in the total soluble protein fraction, again quite different from the profile seen with casein.

That mature casein was being degraded during the re-incubation period was indicated in experiments where *N*-acetylmannosamine was the radiolabelled precursor. Sialic acid, which is derived from this precursor, is a major component of the carbohydrate chains of all five major casein polypeptides in the rabbit (Al-Sarraj *et al.*, 1978b). Again, after an initial increase in radioactivity after the 2 h pulse, radioactivity was lost from casein in a manner and rate similar to the loss of proline radioactivity (Fig. 3). The extent of loss of radioactivity appeared to be not as great as with the amino acid precursors, and

cellular pool of amino acid that will not exchange during the washing procedure with unlabelled amino acid at 4°C. Subsequently the dilution of such a pool

this might well be due to re-utilization of radiolabel derived from degraded casein in the re-incubation period, since we have been unable to remove completely intracellular radioactivity of sugars from mammary tissue after a pulse (B. Speake, D. A. White, N. Paskin & R. J. Mayer, unpublished work).

Cycloheximide immediately inhibits the incorporation of L-[4,5-³H]leucine into proteins of mammary explants (White & Speake, 1980). Addition of the inhibitor immediately after the post-incubation wash in the present experiments prevented both the increase and decrease in radioactivity in casein, indicating that the rise in radioactivity seen in Fig. 2 was due to newly synthesized casein. Inclusion of chloroquine in the re-incubation medium, although not affecting the initial increase in casein radioactivity, i.e. synthesis, completely prevented the subsequent loss of radioactivity from this fraction seen in the absence of the drug. Although probably acting at more than one site in the cell, a major action of chloroquine is to interfere with proteolytic activities of the lysosome (Poole *et al.*, 1978). Its action in preventing casein degradation suggests that in its absence much of the newly synthesized casein is degraded by lysosomal proteinases during the passage of casein from the Golgi to the alveolar lumen. It is probable that degradation occurs after completion of the peptide in the Golgi, since the use of both amino acid and sugar precursors exhibited the same radioactivity profiles on re-incubation and all measurements have been made on mature glycosylated and phosphorylated casein, which focuses at pH 4.6.

The results presented indicate that a major proportion of newly synthesized casein is rapidly degraded intracellularly in mammary explants ($t_{\frac{1}{2}}$ 100 min). Subsequently little casein degradation occurs ($t_{\frac{1}{2}}$ 76 h), agreeing with previous observations by alternative methods of measurement (Wilde *et al.*, 1980). The studies described here constitute the first report of extensive intracellular destruction of a newly synthesized milk protein. Whether this mammary-explant phenomenon occurs during lactogenesis and/or lactation *in vivo* is not known. However, such destruction of casein is similar to that described for other secretory proteins such as collagen (Bienkowski *et al.*, 1978), parathyrin (Morrissey & Cohn, 1979), prolactin (Shenai & Wallis, 1979) and insulin (Halban & Wollheim, 1980). It appears that secretory proteins undergo extensive intracellular degradation irrespective of whether the protein is stored intracellularly in a granular form (e.g. prolactin, insulin and parathyrin)

or as rapidly secreted vesicular material (e.g. collagen and casein). Degradation of such a large proportion of secretory proteins in different cell types affords a marked post-translational, as opposed to a transcriptional, method of controlling the amount of protein secreted.

We thank the Agricultural Research Council and the Iraqi Ministry of Higher Education for financial support for this project. H. R. H. is indebted to the Iraqi Ministry of Higher Education for a research scholarship.

References

- Al-Sarraj, K., White, D. A. & Mayer, R. J. (1978a) *Biochem. J.* **173**, 877–883
- Al-Sarraj, K., White, D. A. & Mayer, R. J. (1978b) *Int. J. Biochem.* **9**, 269–277
- Al-Sarraj, K., Newbury, J., White, D. A. & Mayer, R. J. (1979) *Biochem. J.* **182**, 837–945
- Bienkowski, R. S., Baum, B. J. & Crystal, R. G. (1978) *Nature (London)* **276**, 413–416
- Craig, R. K., Perera, P. A. J., Mellors, A. & Smith, A. E. (1979) *Biochem. J.* **184**, 261–267
- Forsyth, I. A. & Myers, R. P. (1971) *J. Endocrinol.* **51**, 157–168
- Halban, P. A. & Wollheim, C. B. (1980) *J. Biol. Chem.* **255**, 6003–6005
- Harrison, O. S., Craig, R. K. & Campbell, P. N. (1976) *Biochem. Soc. Trans.* **4**, 340–341
- Houdebine, L. M. & Gaye, P. (1975) *Nucleic Acids Res.* **2**, 165–177
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Mercier, J. C. & Gaye, P. (1980) *Ann. N.Y. Acad. Sci.* **343**, 222–251
- Morgan, J. F., Morton, H. J. & Parker, R. C. (1950) *Proc. Soc. Exp. Biol. Med.* **73**, 1–8
- Morrissey, J. J. & Cohn, D. V. (1979) *J. Cell Biol.* **83**, 521–528
- O'Farrel, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Paskin, N. & Mayer, R. J. (1978) *Biochem. J.* **174**, 153–161
- Poole, B., Ohkuma, S. & Warburton, M. (1978) in *Protein Turnover and Lysosome Function* (Segal, H. & Doyle, D., eds.), pp. 43–58, Academic Press, New York
- Shenai, R. & Wallis, M. (1979) *Biochem. J.* **182**, 735–743
- Speake, B. K., Dils, R. & Mayer, R. J. (1975) *Biochem. J.* **148**, 309–320
- White, D. A. & Speake, B. K. (1980) *Biochem. J.* **192**, 297–301
- Wilde, C. J., Paskin, N., Saxton, J. & Mayer, R. J. (1980) *Biochem. J.* **192**, 311–320
- Zak, R., Martin, A. F. & Blough, R. (1979) *Physiol. Rev.* **59**, 407–447