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EFFECTS OF MONENSIN ON THE PROCESSING OF PRO-OPIOMELANOCORTIN IN THE INTERMEDIATE LOBE OF THE RAT PITUITARY

Philippe Crine and Luce Dufour

Département de Biochimie, Université de Montréal, C.P. 6128, Succursale A, Montréal, Québec H3C 3J7, Canada

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SUMMARY: We have studied the effect of monensin on the maturation of pro-opiomelanocortin, the common precursor to α-melanotropin and β-endorphin in intermediate lobe cells of the rat pituitary. Our results show that monensin can completely inhibit the proteolytic processing of pro-opiomelanocortin but does not interfere with the addition of terminal sugars on carbohydrate side chains as tested by their sensitivity to endo-β-N-acetylglucosaminidase H.

The discovery of pro-opiomelanocortin (POMC), the common glycoprotein precursor to adrenocorticotropic hormone (ACTH) and β-endorphin (1-4), has posed intriguing problems including the nature and specificity of the proteases involved in the processing, the role of the carbohydrate side chains and the sites of conversion inside the cell. Studies from several laboratories suggest that cleavage of the precursor may start in the Golgi apparatus (5,6) and is certainly completed in the secretion granule (7,8). On the other hand, glycosylation has been found to involve the cotranslational addition of "high mannose" side chains to asparagine residues (9), the subsequent removal of glucose and mannose residues as the protein moves from its site of synthesis on the rough endoplasmic reticulum to the Golgi apparatus (10) and finally the addition of terminal sugars in the Golgi apparatus (10,11).

Monensin, a monovalent cationic ionophore, has been shown to impede the exit of secretory and membrane proteins from the Golgi apparatus in several

Abbreviations: ACTH: adrenocorticotropic hormone, adrenocorticotropin; POMC: pro-opiomelanocortin; MSH: melanostimulating hormone, melanotropin; SDS : sodium dodecyl sulfate; Endo H: endo-β-N-acetylglucosaminidase H; TCA: trichloroacetic acid. RPMI: Roswell Park Memorial Institute.

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cell types (12-15). This drug is being increasingly used as a tool for studying possible relationships between various intracellular processing events. In the present report, we describe the effect of monensin on the glycosylation and proteolytic processing of POMC by cultured intermediate lobe cells of the pituitary.

MATERIAL AND METHODS

Incubation of intermediate lobe cells: Rat intermediate lobe cell suspensions were prepared as previously published by Mains and Eipper (2). Cells were allowed to attach overnight to poly-L-lysine treated 96-well microculture plates at a density of 40,000 cells per well. For continuous labeling, the cells were routinely incubated at 37°C with monensin (a generous gift from Dr. R.L. Hamill, Lilly Research Laboratories, Indianapolis) for 1 h prior to the addition of [^{3}H] leucine (Amersham Corporation, 130 Ci/mmol; 100 µCi/ml). During pulse-chase experiments, intermediate lobe cells were first incubated for 10 min in phenylalanine-free RPMI medium supplemented with [^{3}H]phenylalanine (Amersham Corporation, 90 Ci/mmol 2 mCi/ml), washed in complete RPMI medium supplemented with 2 mM non-radioactive phenylalanine and then incubated 2 h longer in the presence or the absence of 10 µM monensin (chase).

Extraction of pituitary peptides and their analysis on SDS-polyacrylamide gel electrophoresis was done as described earlier (1,16). The radioactivity in 10% trichloroacetic acid precipitates was measured according to the procedure of Mans and Novelli (17).

Digestion with endo-β-N-acetylglucosaminidase H: For endo-β-N-acetylglucosaminidase H (Endo H) digestion, cells were first extracted by three cycles of freezing and thawing in 5N acetic acid containing 2 mM phenylmethylsulfonyl fluoride and 2 mM iodoacetamide as protease inhibitors. After lyophilization, the samples were dissolved in 0.2 M ammonium acetate pH 5.0 and incubated with or without 5 m U of Endo H (30 U/mg : Miles Biochemicals) for 3 h at 37°C. After the incubation, the samples were lyophilized, redissolved in the electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

RESULTS AND DISCUSSION

A dose titration curve was first obtained to study the effect of monensin on protein synthesis by cultured intermediate lobe cells. As shown in fig. 1A, monensin concentrations as low as 0.1 µM inhibited total intermediate lobe protein synthesis by 50% (Fig. 1A). At a concentration of 0.5 µM, the effect of monensin on protein synthesis was maximum (65% inhibition) and further increases of the ionophore concentration did not result in any additional inhibition of protein synthesis. Inhibition of protein synthesis was not due to cell death since no significative difference in cell viability was

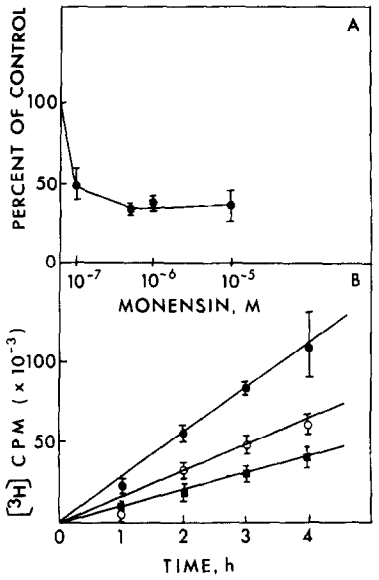

Figure 1. Effect of monensin on the synthesis of intermediate lobe proteins. In (A) incubation was for 16 h with monensin added at the concentration indicated. In (B) incubation was for the time indicated, with monensin added at a concentration of 10^{-5} M (■), 10^{-7} M (○) or with no monensin (●). Protein synthesis was assessed by measuring [^{3}H] leucine incorporation into TCA precipitable proteins.

observed after a 16 h monensin treatment at all concentrations tested. Moreover, even at the highest monensin concentration tested, the rate of protein synthesis remained linear for at least 4 h (fig. 1B). Inhibition of procollagen and fibronectin synthesis has already been observed by Uchida et al. (14) who postulate the existence of a feedback phenomenon shutting off the synthesis of secretory proteins as they accumulate in Golgi structures. This feedback effect could explain the partial inhibition of protein synthesis observed at low ionophore concentrations. In cells treated with higher concentrations, we cannot exclude that an eventual uncoupling of oxidative phosphorylation could cause a depletion of the cellular pool of ATP and therefore result in an overall inhibition of protein synthesis. In order to not interfere with the synthesis of POMC during subsequent pulse-chase experiments, monensin was added only at the beginning of the chase-incubation.

Previous studies had shown that during pulse-chase incubations of intermediate lobe cells, radioactive precursor forms initially synthesized

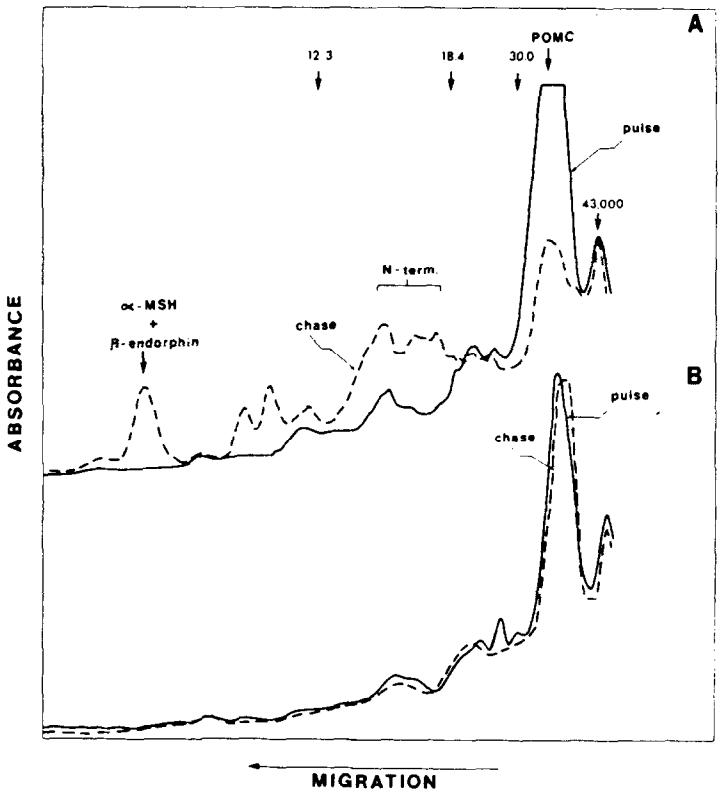

Figure 2. Effect of monensin on POMC proteolytic processing. Intermediate lobe cells were first pulse-incubated for 10 min in RPMI medium containing [^{3}H] phenylalanine and then chased for 2 h in the absence of monensin (A) or with 10 µM monensin (B). Pulse and pulse-chase samples were then analyzed by electrophoresis in 8.4 M area, 0.1% SDS polyacrylamide gels (16). Fluorograms were scanned with a microdensitometer. Each sample contained approximately 3.5 x 10^5 cpm. Exposure time for fluorography was 4 h in B. In A, exposure time was extended to 16 h in order to obtain satisfactory contrast for lower molecular weight peptides. Under those conditions however overexposure of the POMC band in the "pulse" sample was observed. POMC and the various maturation products were identified as described earlier (1,16). The position of ^{14}C-labeled proteins (from New England Nuclear) is shown as a reference molecular weight scale. (—) pulse; (---) chase.

during the pulse could be cleaved during a 2 h chase into maturation products identified as α-MSH, β-endorphin and variant forms of the N-terminal glycopeptide (1,16). A typical analysis of these peptides on polyacrylamide gel is illustrated in fig. 2A. When monensin (final concentration : 10 µM) was added at the beginning of the chase incubation, complete inhibition of the maturation process was observed (fig. 2B). Oda and Ikehara (18) have recently published similar results on the inhibition of proalbumin processing and suggested that monensin arrests the intracellular transport of proalbumin before the site where its conversion takes place. Alternatively, monensin

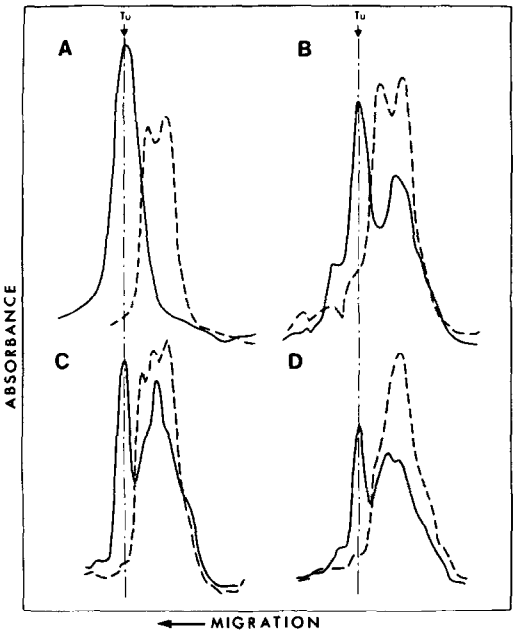

Figure 3. Endo H digestion of POMC. Intermediate lobe cells were pulse-incubated for 10 min in RPMI medium containing [^{3}H] phenylalanine and then immediately processed for analysis by gel electrophoresis (A) or further chase-incubated for 30 min without monensin (B) or with 10 µM monensin (C). In D, the chase-incubation was for 2 h with 10 µM monensin. Cells were then extracted in 5 N acetic acid and digested with 5 mU of Endo H as explained under "Experimental Procedures". Control incubations were performed without Endo H. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and the fluorograms were scanned with a microdensitometer. The position of POMC forms synthesized in the presence of tunicamycin (Tu) is marked as a reference (20). (——) Endo H treated; (--) No Endo H.

being a cationic ionophore, could alter the cation concentration or raise the pH inside intracellular membranes. There is evidence that the POMC maturation enzyme requires low pH (7). Inhibition of POMC processing by monensin could therefore result from a direct inhibition of the processing enzyme itself.

The next experiments were designed to determine whether monensin interfered with terminal glycosylation in the Golgi apparatus. When POMC forms, synthesized during 10 min pulse-labeling incubations of intermediate lobe cells, were digested with Endo H and analyzed by SDS polyacrylamide gel electrophoresis, they comigrated with the nonglycosylated peptides synthesized in the presence of tunicamycin, slightly ahead of normal fully glycosylated forms (fig. 3A). When a 30 min chase was performed in the absence of ionophore (fig. 3B) or with 10 µM monensin (fig. 3C), some of the precursor molecules

remained as high molecular weight species after Endo H digestion, and were therefore thought to have acquired terminal sugars. Planimetric estimation of the area under the different peaks of fig. 3 showed that terminal sugar addition is limited to approximately 65% of the carbohydrate side chains during the first 30 min of the chase incubation and that monensin did not interfere significantly with this kind of post-translational modification. Extension of the chase-incubation to up to 2 h in the presence of monensin did not produce additional terminal glycosylation (fig. 3D).

This observation is somewhat at variance with the results of Tartakoff and Vassalli (19) who concluded that monensin prevents the labeling of newly synthesized immunoglobulin molecules with tritiated terminal sugars. However, Strous and Lodish (15) have found that in the same cell, processing of two different glycoproteins (the secretory protein transferrin and the protein G of Vesicular Stomatitis Virus) could be blocked by monensin before (for transferrin) or after (for the protein G) the addition of terminal su---

In conclusion, we have shown that, in intermediate lobe cells, monensin completely blocks proteolytic processing of POMC but has very little effect on glycosylation. For this reason, and despite the fact that its mechanism of action is still poorly understood, we believe that this ionophore could serve as a useful tool for studying the relationship between the various posttranslational processing events involved in the maturation of the prohormone.

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