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FURTHER CHARACTERIZATION OF THE GLUCOCORTICOID-INDUCED ANTIPHOSPHOLIPASE PROTEIN "RENOCORTIN"

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The steroid-induced anti-phospholipase protein "Renocortin" has been further characterized by column chromatography and a monoclonal antibody. In medium devoid of foetal calf serum, renomedullary insterstitial cells in culture exposed to the anti-inflammatory steroid dexamethasone released 3 peptides of apparent MW : 15k, 30k, and 45k possessing similar biological properties to "renocortin", "lipomodulin" and "macrocortin". A monoclonal antibody directed against macrocortin also bound the 45k peptide released from the renomedullary cells. The 15k species was, like macrocortin, inactive in an "in vitro" enzymatic assay but recovered its full inhibitory activity after dephosphorylation by alkaline phosphatase treatment. We conclude that "macrocortin", "lipomodulin" and "renocortin" are similar if not identical proteins. We propose a scheme to account for "in vivo" secretion and regulation of these proteins.

INTRODUCTION Anti-inflammatory steroids reduce prostaglandin synthesis in intact cells (1,2,3) and in isolated organs (4,5,6) by inhibiting the release of the polyunsaturated fatty acid precursors (7-10) for both cyclo-oxygenase and lipoxygenase pathways. Among the various biological activities exerted by glucocorticoids in the organs and tissues of mammals, their acute anti-inflammatory activity is now related to the induction of phospholipase inhibitory proteins responsible for this inhibition of arachidonic acid release (11-13). These proteins have been isolated from glucocorticoid-treated rabbit neutrophils (lipomodulin with MW : 40k (11)), rat macrophages (macrocortin with MW : 15k (12)), and rat renomedullary interstitial cells in culture (renocortins with MW :15 and 30k (13)). All these proteins exhibit similar biological activities in that they inhibit arachidonate release ; both lipomodulin and macrocortin also exert anti-inflammatory activity on carrageenan-induced paw oedema and pleurisy (14-15). Recent data reported by Hirata et al (16) have demonstrated that macrocortin, (the 15k peptide) was probably a phosphorylated fragment of the 40k peptide "lipomodulin". Until now, there was no evidence that renocortin was related to any of these other proteins, although they shared the same

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biological properties with respect to arachidonic acid release and phospholipase A2 activity. We now report that dexamethasone treated reno-medullary interstitial cells in culture medium devoid of Foetal Calf Serum (FCS), release 3 peptides of apparent MW : 15k, 30k, 45k possessing the same biological properties of "Renocortin". We also report evidence that "renocortin", "macrocortin" and "lipomodulin" are similar if not identical proteins, the 45k peptide being under certain conditions cleaved to 2 smaller fragments of 30k or 15k. We also suggest a possible scheme of secretion for these proteins "in vivo".

METHODS

Cell culture and cell line Monolayer tissue cultures of rat renomedullary interstitial cells were obtained by the technique previously described (3). The cell line was maintained to the 45th passage. Under similar culture conditions, these cells were shown (17) to retain the characteristics of medullary interstitial cells. Cells were grown in RPMI 1640 medium supplemented with 10 % FCS, unless otherwise specified.

Treatment of cells with dexamethasone Cells were grown for 24 h after seeding in 10 ml of fresh culture medium plus 10 % FCS. The medium was then removed for assay of basal prostaglandin E_2 (PGE_2) content and replaced by 10 ml of fresh culture medium. Dexamethasone 10^{-7}M (final concentration) was added to some flasks. After an additional 24 h incubation period, medium from control and dexamethasone treated flasks was collected. An aliquot of all supernatants was assayed for PGE_2 in order to determine the initial inhibition produced by dexamethasone. Cells were then rinsed thoroughly three times with Hanks balanced salt solution, in order to eliminate all FCS, and the cells were incubated for an additional 5 h in RPMI 1640 alone. We had previously (10) demonstrated that under our culture conditions, the protein was released for 22 h after steroid removal. After this second incubation period, supernatants from control and dexamethasone treated cells were collected. As before, an aliquot of all supernatants was assayed for the determination of the inhibitory effect on PGE_2 secretion. Cells were rinsed again and, with a rubber policeman, suspended in phosphate buffer saline, containing 2mM EDTA . These cells were then disrupted by ultrasonication using a "Ultrasonics" cell disruptor. After centrifugation, the membranes were solubilized with a non ionic detergent (Nonidet P40) for 20 min at 4°C and centrifuged at 105,000 g for 1 h and the high speed supernatants collected.

The following experiments were done using either supernatants from dexamethasone treated cells containing 10 % FCS, or supernatants without FCS, or high speed supernatants from cell extracts (containing the solubilized membrane proteins).

Gel Permeation Chromatography All supernatants from control and dexamethasone treated cells were separated by gel permeation on an Ultrogel AcA54 column (IBF reagents, Villeneuve la Garenne, France). The column was equilibrated and eluted with ammonium acetate buffer 100mM, pH 7.4 , and the void volume determined with blue dextran. Calibration of the column was performed utilizing proteins of known molecular weights. Fractions were collected, lyophilized, dissolved in RPMI 1640 and tested either for their ability to inhibit PGE_2 secretion over a 40 min incubation period in untreated cells or for their ability to inhibit soluble phospholipase A_2 activity (as described in the next paragraph).

Biological identification of the fractions

Inhibition of prostaglandin E_2 secretion or cell assay Cells were grown for 24 h after seeding in 24 multiwell Linbro plates with 1 ml

of medium + 10 % FCS. Medium was discarded and replaced by 0.5 ml of the samples for testing. After a 40 min incubation period, supernatants were recovered and PGE_2 synthesis assessed in all samples after extraction by the radioimmunoassay procedure previously described (3, 18). Cells were then rinsed with Hanks balanced salt solution, dissolved in 1N NaOH and the cellular protein content measured according to the method of Lowry et al (19). The secretion of PGE_2 was expressed as ng of PGE_2 per µg of cellular protein. Results were expressed as percentage inhibition of PGE_2 produced by supernatants derived from dexamethasone treated cells versus supernatants derived from control cells

Inhibition of phospholipase A_2 activity or enzyme assay Membrane phospholipids of E.Coli were labeled by incorporating [^{3}H]oleic acid (10 Ci/mmole, Amersham U.K.) into their medium during their exponential growth phase. The labeled E.Coli was then killed by autoclaving and recovered by centrifugation, washed repeatedly in Tris buffer 200 mM pH 8 containing 10 mg/ml bovine serum albumin. When the washings contained negligible radioactivity , this substrate was stored at 4°C in the same buffer + 0.1% sodium azide and used as required as an exogenous substrate for the phospholipase A_2 assay. Therefore, the labeled substrate was [^{3}H] oleic acid labeled E.Coli , containing approximately 3.10^6 cpm / ml. The incubation was carried out in Tris buffer 100mM, pH 8, containing 10 mM $CaCl_2$, 50 ng porcine pancreatic phospholipase A_2 (Sigma Chemical Corp.), with or without the fraction to be tested, in a final volume of 350 µl. After a 10 min preincubation period at 4°C with the enzyme alone, the reaction was started by addition of 50 µl labeled E.Coli suspension and allowed to incubate for a further 5 min at 4 ° C. The reaction was then stopped by adding 100 µl 2N HCl , an excess of bovine serum albumin (100 µl containing 100 mg/ml) was added to the reaction tube to trap the fatty acids liberated and the tubes were centrifuged for 5 min at 10,000g. An aliquot of the supernatant, which contains the liberated free [^{3}H]oleic acid, and therefore reflects PLA_2 activity, was counted by liquid scintillation spectrometry.

Immunological experiments with the fractions A monoclonal antibody raised against macrocortin, Rm23 (20) was tested on the various fractions which had been separated as previously described. The biologically active fractions were first incubated together with the antibody at an appropriate dilution for 30 min at 4°C , and the phospholipase A_2 assay was then performed as described above.

Dephosphorylation experiments Various fractions were treated with insoluble alkaline phosphatase (Sigma, 1U/tube, 30 min at 25°C) after which the tubes were centrifuged in order to remove the enzyme and the phospholipase A_2 assay was performed on the supernatant, as described previously.

RESULTS

Peptide separation in supernatant containing 10 % FCS In all the following experiments, unless otherwise specified, the biological activity of the effluent fractions was assessed by the inhibition of prostaglandin secretion produced in untreated cells after a 40 min incubation period. In the effluent fractions of the supernatants derived from dexamethasone treated cells, inhibitory activity of PGE_2 secretion in untreated cells was found in two zones corresponding to apparent molecular weights of 15 k and 30 k. No inhibitory activity was found in the 45 k zone (Fig. 1a). These results have already been reported previously. (13)

Peptide separation in supernatant devoid of FCS Supernatants derived from dexamethasone-treated cells cultured without FCS contained the same 15 and 30 k inhibitory peaks, but under these conditions, another major

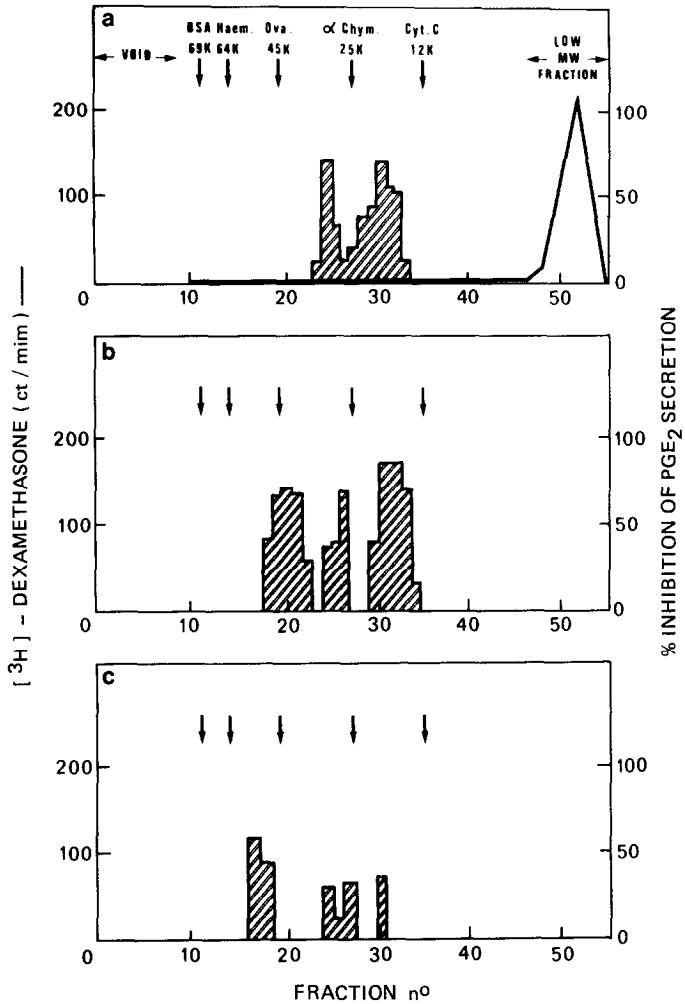

Figure 1a Gel permeation experiments on an ultrogel AcA column of supernatants containing 10% FCS derived from cells treated with dexamethasone 10^{-7} M for 24 h. Void volume was determined by the elution of dextran blue, dexamethasone eluted with low molecular weight fractions. The column was calibrated by measuring the elution volume (Ve) of bovine serum albumin (69k), haemoglobin (64k), ovalbumin (45k), alphachymotrypsin (25k) and cytochrome C (12k). The inhibition of PGE_2 secretion during a 40 min incubation period in untreated cells is shown as a histogram (hatched columns).

Figure 1b The same experimental procedure as in Fig 1a was followed but the fractions studied were derived from the supernatants of the same cells as in Fig 1 but devoid of FCS.

Figure 1c The same experimental procedure as in Fig 1a was followed but the fractions studied were derived from NP40 solubilized cell membrane extracts obtained from the same cells as in Fig 1a and 1b.

peak possessing biological activity was found in the 45 k fraction (Fig. 1b).

Peptide separation in the cell extract In fractions derived from the cell extracts (containing the solubilized membrane proteins), obtained

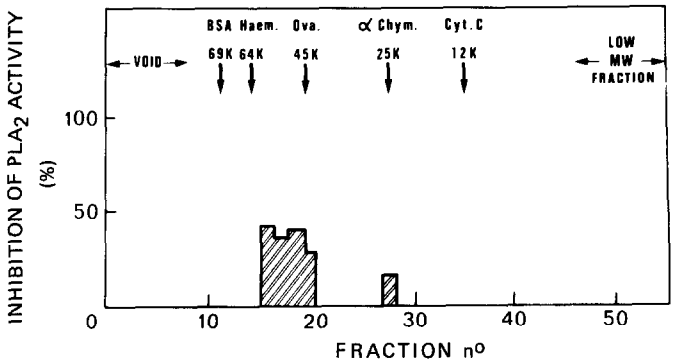

Figure 2 The same fractions as in Fig 1b (derived from the supernatants of dexamethasone-treated cells, devoid of FCS) were tested for their ability to inhibit porcine pancreatic phospholipase A2 activity. The inhibition of phospholipase A2 activity (expressed as the % of inhibition found in the samples versus buffer alone) is shown as a histogram (hatched columns).

from dexamethasone treated cells, prepared as described above, we also found 3 peaks possessing biological activity, in the 15, 30 and 45 k molecular weight zones (Fig. 1c).

Antiphospholipase A_2 activity of the different peptides When the inhibitory fractions which had been tested in the cell assay, were tested in the enzyme assay, similar biological activities were found in the 30 k and the 45 k zones ; the 15 k zone on the contrary was inactive in this enzyme assay (Fig. 2).

Dephosphorylation of the 15 k peptide The 15 k peptide was dephosphorylated by treatment of the fraction with insoluble alkaline phosphatase for 30 min at room temperature, and then tested for its ability to inhibit phospholipase A_2 activity in the enzyme assay. After such a treatment, this fraction recovers its full inhibitory activity.

(Before treatment, no inhibition, after treatment, the inhibition is of 37.2 ± 7.9 % n=3).

Immunological identification of the different peptides When the different fractions were treated with the monoclonal antibody Rm 23, the fractions which were active loose a substantial part of their activity after only 30 min incubation with the antibody. (see Table 1).

DISCUSSION

1) In a previous paper (13), we had demonstrated that anti-inflammatory steroids were able to induce in cultured renomedullary interstitial cells, the synthesis of "renocortins", two polypeptides possessing anti-phospholipase properties. These induced proteins could be responsible for part of the anti-inflammatory actions of corticosteroids. Using the same model, but a different experimental approach, we report in this paper, that our cells, when treated by dexamethasone release and synthesize 3 polypeptides possessing the biological properties of "renocortins". The molecular weights of these peptides are not only the

Table 1

Fraction	Inhibition (%)	n
45k	48.2 ± 9.5 *	3
45k + Ab	18.1 ± 4.3 *	3
30k	58.8 ±10.7 +	3
30k + Ab	29.4 ± 0.2 +	3

The fractions corresponding to molecular weights of 30k and 45k , inhibited porcine pancreatic phospholipase A2 activity but lost their activity when they were treated with the monoclonal antibody Rm 23 at a dilution of 1:100 for 30 min at 4°C. Results are expressed as % inhibition of the sample versus buffer alone (mean ± s.d.).

* : $p < 0.01$
+ : $p < 0.01$ (student's t test)

15k and 30k species, as reported in the previous report but also a 45k protein. Our results were therefore very similar to those reported by Hirata et al (11) and by Flower et al (12), allowing us to speculate that "macrocortin", "lipomodulin" and "renocortin" were in fact the same proteins. Why were two peptides found in culture medium containing 10 % FCS, whereas three peptides were found in the supernatants of serum free medium ? We postulate that FCS contains a protease which cleaves the 45k peptide either into 2 active peptides of 30k and 15k, or into an active 30k peptide which is subsequently cleaved to an active 15k peptide.It seems likely that under physiological conditions, in plasma, the 45k peptide is released but split into the smaller fragments. This hypothesis had been already postulated by Hirata et al (16) for lipomodulin.

2) Using both cell and enzyme assays to test the same fractions, we found a certain discrepancy in our results. In the cell assay, we found 3 species (45k, 30k and 15k) but using the enzyme assay, we could only find 2 species (45k and 30k). A similar discrepancy had already been noticed by Hirata et al . He suggested that the smaller peptide (15k) was circulating in a phosphorylated form and that the enzyme assay could not detect this species. In our model, we found similar results, since the 15k peptide recovered its inhibitory activity in the enzyme assay after it had been treated with alkaline phosphatase. In our cell assay, the 15k peptide was found very active. Since many cells contain alkaline phosphatase on their surface (22), we postulated that in our cell assay, the 15k fragment was dephosphorylated and then rendered active.

3) The monoclonal antibody Rm 23 which has been already been shown to bind to lipomodulin (F.Hirata, personal communication), also cross reacts with our peptides and inhibits their biological activity suggesting that renocortin is similar to lipomodulin and to macrocortin. These proteins share also the same mode of synthesis, (i.e.induced by glucocorticoid treatment), the same biological properties, the same molecular weights, and the same cross-reactivity with monoclonal antibodies against either lipomodulin or macrocortin. Therefore, we

conclude that we are probably dealing with the same proteins, and that this pathway of steroid action is not reserved only for inflammatory cells (neutrophils and macrophages). This phenomenon would seem to be of a more general physiological relevance. Further experiments are required to elucidate the exact role of these proteins.

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