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PURIFICATION AND PARTIAL SEQUENCING OF INHIBITORY FACTOR ON RENAL MEMBRANE ADENYLATE CYCLASE IN PANCREATIC CANCER EXTRACT : IDENTITY WITH HISTONES H1B OR H1D

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SUMMARY: Inhibitory activity on renal membrane adenylate cyclase (AC) has previously been found in the extract of a pancreatic cancer associated with humoral hypercalcemia of malignancy (HHM). AC inhibitor was purified employing inhibition of AC activity of renal membrane stimulated by forskolin as its index. N-terminal 9 residues and a digested fragment of purified protein (14 residues) were completely consistent with that of histones H1b and H1d. Not only histone H1 but also histones H2A, H2B and H3 from calf thymus inhibited AC activity. These results indicate that the AC inhibitor in the pancreatic cancer extract is histone H1b or H1d and histones H2A, H2B and H3 also have an AC inhibitory activity.

Investigation of the pathogenesis of humoral hypercalcemia of malignancy (HHM) has recently resulted in the isolation of a parathyroid hormone-related protein (PTH-rP) from human neoplasm. It is structurally similar to parathyroid hormone (PTH) in the aminoterminal region (1-4) and interacts with PTH receptor to stimulate both renal and skeletal adenylate cyclase (AC) (5). We have also reported that pancreatic cancer associated with HHM produced a PTH-like factor which increases cAMP level both in primary culture from rat calvaria and osteogenic cell line, MC3T3E1 (6). However, in renal membrane, partially purified extract of the pancreatic cancer with a PTH-like activity did not stimulate but rather dose-dependently inhibited AC activity stimulated by human(h) PTH(1-34), PTH-rP(1-34), isoproterenol,

Abbreviations used: AC, adenylate cyclase; HHM, humoral hypercalcemia of malignancy; PTH, parathyroid hormone; PTH-rP, parathyroid hormone-related protein; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; GnRH-BI, gonadotropin-releasing hormone-binding inhibitor.

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vasoactive intestinal peptide(VIP), cholera toxin, pertussis toxin, or forskolin (7). As the tumor extract readily stimulated cAMP production of renal cortical slices and further enhanced the effects of submaximal doses of PTH and forskolin to stimulate cAMP production (7), we reasoned that the tumor extract simultaneously contained the stimulatory activity in cAMP production of intact cells and an inhibitory activity on AC of broken cell preparation.

In the present study, the AC inhibitory factor in the tumor extract was identified and purified. Inhibition of AC activity of renal membrane stimulated by forskolin was examined as the index of AC inhibitory activity. It was found that the AC inhibitor was identical with histones H1b and H1d in the partial amino acid sequences. AC inhibitory activity was also observed when the membrane was incubated with histones H1, H2A, H2B and H3.

MATERIALS AND METHODS

Preparation of tumor extract

Tumor tissue obtained at autopsy from a well characterized case of exocrine pancreatic cancer associated with humoral hypercalcemia of malignancy was stored at -80℃ and subjected to urea extraction followed by ethanol sodium-chloride fractionation as described by Burtis et al (8). The crude extract was chromatographed employing the LKB Ultrochrom GTi system. Reverse phase high performance liquid chromatography (HPLC) was first carried out using a BENSIL 7-C8 column (BENTEC Ltd., Chiba, Japan) as described previously (7). Active fractions containing PTH-like and AC inhibitory activities were pooled and rechromatographed on a Vydac 218 TP 54 C18 column by increasing the concentration of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a rate of 0.1%/min. Some fractions containing AC inhibitory activity were reconstituted in 20mM sodium phosphate containing 25% acetonitrile and applied to a Mono S HR5/5 cation exchange column. The column was eluted with a linear gradient from 0 to 1M sodium chloride.

Assay of AC activity

The cAMP generation of a clonal osteogenic cell line, MC3T3E1, was examined as indices of PTH-like activity. AC activity of canine renal cortical plasma membrane prepared by sucrose gradient ultracentrifugation (9) was assayed as described previously (7). cAMP was measured by RIA (Yamasa Shoyu Co., Chiba, Japan).

SDS-PAGE

The purified proteins were electrophoresed on a 14-18% sodium dodecylsulfate (SDS) polyacrylamide gel using Pharmacia LKB Biotechnology Phast system. Samples were boiled for 3 min in 2% SDS, 5% β-mercaptoethanol, Tris-HCl(pH 8.0) and applied on the gel. After electrophoresis, the gels were stained by silver.

Amino acid sequence analysis

Amino acid sequences of N-terminal and a digested fragment were analyzed by a gas-phase sequencer (model 470A, Applied Biosystems. Phenylthiohydantoin derivatives of amino acid were identified as described previously (10). Lyophilized proteins

from salt free solutions were dissolved and digested with staphylococcus aureus V8 protease for 5.5 hours at 36℃ in 4M urea, 50mM ammonium bicarbonate, pH 7.8. The digested proteins were peptide-mapped by reverse phase HPLC as described previously (10).

Synthetic hPTH(1-34) was kindly donated from Toyo Jozo Co., (Tokyo, Japan). Calf thymus histones (H1, H2A, H2B, H3) and myelin basic protein (from bovine brain) were purchased from Sigma Chemical Co.(St. Louis, MO), and forskolin was from Calbiochem Corporation (La Jolla, CA). Statistic analyses were performed by Student's t-test.

RESULTS

PTH-like activity in the pancreatic cancer extract, defined as a stimulating activity on cAMP production in MC3T3E1 cell, was eluted in two peaks by reverse phase HPLC at 31% and 34% of acetonitrile as described previously (7). The inhibitory activity on AC stimulated by forskolin in renal membrane was eluted very close to or concomitant with PTH-like activity on reverse phase HPLC. These fractions were pooled and rechromatographed on C18

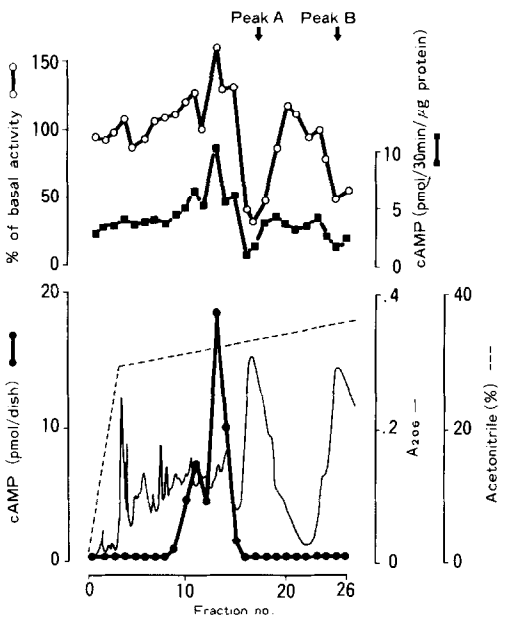

Fig. 1. Profile on reverse phase HPLC of AC inhibitory activity in renal membrane (top and middle) and AC stimulating activity in MC3T3E1 cells (bottom). Tumor extract was first chromatographed on a C8 column, and fractions which had PTH-like activity and AC inhibitory activity were pooled and rechromatographed on a Vydac 218 TP54 C18 column. Proteins were eluted by increasing the concentration of acetonitrile from 29 to 36% in 0.1% TFA over 70 min at a flow rate of 0.4 ml/min, and 1.2 ml fractions were collected. After lyophilization, each fraction was redissolved in 400 μl 0.001 N acetic acid. The aliquots used for assays were: 5 μl for cAMP response of MC3T3E1 cells (bottom), 5 μl for cAMP response of renal membrane in the presence (top) or absence (middle) of forskolin ($2x10^{-6}$ M). 100% denotes the AC activity in the presence of forskolin alone.

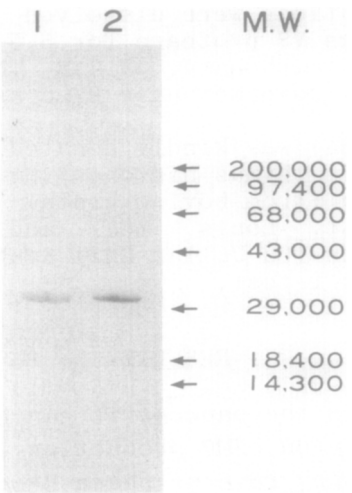

Fig. 2. SDS-PAGE of purified peak A and peak B. PAGE and silver staining was carried out as described. Lane 1, purified peak A ; lane 2, peak B. Molecular size markers are indicated in the right side.

reverse phase HPLC (Fig. 1). The AC stimulating activity of renal membrane was detected in the same fraction as the cAMP production stimulating activity in MC3T3E1 cells. Moreover, AC inhibitor was eluted in two peaks at 33% (peak A) and 36% (peak B) of acetonitrile, with no PTH-like activity. Following purification of peak A by cation exchange chromatography, purified protein and peak B were electrophoresed on SDS-PAGE under reducing condition (Fig. 2). Silver-staining of SDS-PAGE revealed that both fractions contained a single band of protein.

Amino acid sequences of peak B were determined by gas-phase sequencing. The protein was not N-terminally blocked and the N-terminal 9 residues of the protein were identical with human spleen histones H1b and H1d (Fig. 3). Moreover, a sequence of digested fragments of peak B was identical with the residues 52-65 of human spleen histones H1b and H1d (data not shown).

AC inhibitory activities of histones H1, H2A, H2B, H3 and myelin basic protein were examined (Fig. 4). All histone families but myelin basic protein dose-dependently inhibited AC activity.

DISCUSSION

We reported previously that the extract of pancreatic cancer associated with HHM contained a PTH-like activity which increased cyclic AMP content in osteoblastic cells along with an AC

	1	2	3	4	5	6	7	8	9
Human spleen histone H1b	Ser	Glu	Thr	Ala	Pro	Ala	Ala	Pro	Ala
Human spleen histone H1d	Ser	Glu	Thr	Ala	Pro	Ala	Ala	Pro	Ala
Peak B	Ser	Glu	Thr	Ala	Pro	Ala	Ala	Pro	Ala

Fig. 3. Comparison of the amino acid sequence of human spleen histone H1b, H1d and AC inhibitor in peak B.

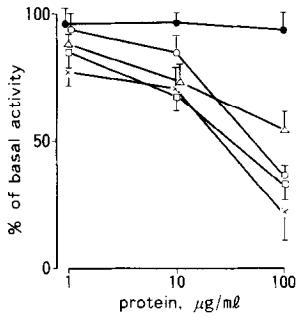

Fig. 4. AC inhibitory activity of renal membranes in the presence of histones H1(○), H2A(△), H2B(×), H3(□) and myelin basic protein(●). 100% denotes the AC activity in the presence of forskolin alone. Values are the mean ± SD of three determinations.

inhibitory activity which was only effective in the broken cell preparation (7). Partially purified tumor extract inhibited AC activity stimulated by either hPTH(1-34), isoproterenol, VIP, cholera toxin, pertussis toxin or forskolin. These results indicated that the AC inhibitor modulated the catalytic site of AC. In the present study, the AC inhibitor was separated from PTH-like activity using reverse phase HPLC and cation exchange chromatography.

Within the limit of the investigation, N-terminal 9 residues and a digested fragment (14 residues) of peak B were completely consistent with histones H1b and H1d(15,16). Ohe et al. described that the fraction of human spleen histone H1 is separated into four subfractions, histones H1a, H1b, H1c and H1d(11-14). Their subfractions have very similar hydropathy profiles, amino acid sequences and molecular weights of about 22,000 daltons. However, the apparent molecular size appeared to be 32,000 dalton on SDS-PAGE due to the influence of their extreme negative charge (pI of histone H1 is 10.5). SDS-PAGE showed that peak B had the same electrophoretic mobility as histone H1. Histone H1 purified from calf thymus also showed an AC inhibitory activity. These results indicate that AC inhibitor included in peak B is probably histone H1b or H1d. However, further study is needed to clarify whether they are the same protein or not, because N-terminal serine of peak B is not acetylated as that of histones H1b or H1d. Moreover, it cannot be entirely excluded that a minute contamination of a strongly active inhibitor(s) at a very low concentration in the purified tumor extract and the commercially available calf thymus histone H1. Until now, only a $\beta\gamma$-subunit of G protein has been reported to directly inhibit the catalytic site of AC (17). However, the amino acid sequence of the purified protein is different from that of $\beta\gamma$-subunit of G protein (18-20). The concentration of $\beta\gamma$-subunit of G protein at amount too minute to be demonstrated by silver staining cannot explain the inhibitory activity.

Histones H2A, H2B and H3 (respective pI : 10.5, 11.0 and 11.0) also have an AC inhibitory activity. They have no

homologous region to histone H1 in amino acid sequences, so the AC inhibitory activity might be mediated by some nonspecific interaction between positively charged proteins and negatively charged plasma membrane. However, another basic protein, myelin basic protein, whose molecular weight is 17,000 dalton and pI is 10.6, did not inhibit AC activity within the comparable concentration of histones. Therefore the mechanism of AC inhibitory action of histones cannot be solely explained by their positive charge and needs to be clarified.

Although this is the first report on histone H1 demonstrating an action outside the nucleus, inhibitory activities of histone families other than histone H1 have been reported on the biological activities of several peptide hormones. A gonadotropin-releasing hormone binding inhibitor(GnRH-BI) from bovine ovaries was purified and identified as histone H2A (21). Both GnRH-BI and histone H2A inhibit luteinizing hormone-stimulated cAMP accumulation in luteal cells and follicle stimulating hormone-induced cAMP and progesterone production in granulosal cells through the mechanism of their inhibitory effects on GnRH binding to rat ovarian membrane(22). A thymic extract, functionally defined as homeostatic thymic hormone by Reichhert et al.(23), which inhibits the responses of adrenocorticotropic, thyrotropic, and gonadotropic hormones, and augments the response to growth hormone in rats, was purified and identified as a mixture of histones H2A and H2B. It is not clear whether such functions of histones are of any physiological relevance or only experimental observations due to artificially released histones during the preparation of tissue extract. However, histones were found on the surface of the cells (24) and in serum and milk (25), and Aten and Behrman (22) proposed a notion that under conditions of cell lysis, such as in follicular atresia and lysis of the corpus luteum, histones might be released in concentrations sufficient to block ovarian cell responses to gonadotropins. It may be suggested that histones might function not only as a core protein of nucleosomes but as a modulator as well, but more study is needed before discussing possible biological significance of the present observation, especially its relation to pathogenesis of HHM.

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