Bone and Kidney Adenylate Cyclase-stimulating Activity Produced by a Hypercalcemic Canine Adenocarcinoma Line (CAC-8) Maintained in Nude Mice

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ABSTRACT

The tumor line, CAC-8, is a serially transplantable adenocarcinoma maintained in nude mice which originated from a hypercalcemic dog. Nude mice with CAC-8 developed a syndrome of humoral hypercalcemia of malignancy. CAC-8 contained a protein factor which stimulated adenylate cyclase of bone and kidney cells in vitro. The adenylate cyclase (AC) of rat osteosarcoma cell lines, ROS 17/2.8 (ROS) and UMR-106, was stimulated by the tumor extract and potentiated by forskolin (0.1 μM). The ROS cells responded to the lowest concentration of CAC-8 extract, but UMR cells responded with a greater increase in AC activity compared to controls following exposure to CAC-8 extract. Pretreatment of ROS 17/2.8 cells with dexamethasone enhanced the response to CAC-8 extract. The opossum kidney cell line (OK) was less sensitive to the AC-stimulating activity of CAC-8 extract, but AC stimulation was increased in the presence of forskolin. Bovine (1-34) parathyroid hormone (BPTH) (10 nM) stimulated AC equally in ROS, UMR, and OK cells. Isoproterenol (1.0 μM) stimulated AC activity in ROS and UMR cells, but not in OK cells. The AC-stimulating activity of CAC-8 appeared to bind to the parathyroid hormone receptor of ROS, UMR, and OK cells since addition of the parathyroid hormone receptor antagonist, [3H]norleucine, [3H]tyrosine]BPTH (3-34) amide, inhibited CAC-8-mediated cyclic adenosine 5'-monophosphate production and alone did not stimulate AC activity. The AC-stimulating activity of CAC-8 was acid and heat stable. Trypsin digestion reduced BPTH and CAC-8 stimulation of AC to near basal levels and treatment of CAC-8 extract with dithiothreitol reduced AC stimulation in UMR cells by approximately 50%. Extracts of the hypercalcemic tumor line (CAC-8) contained bone and kidney AC-stimulating activity which was enhanced by forskolin and dexamethasone, inhibited by [3H]norleucine, [3H]tyrosine]BPTH (3-34) amide, heat stable, trypsin sensitive, inactivated by reduction, and had a relative molecular weight of 34,000 by gel exclusion chromatography. Isolation and characterization of the factor(s) produced by CAC-8 that stimulate AC activity will be useful in further understanding the pathogenesis of humoral hypercalcemia of malignancy in animal and human patients.

INTRODUCTION

A serially transplantable tumor line, designated CAC-8, has been developed from a canine hypercalcemic adenocarcinoma (1) in nude mice that develop an identical clinical syndrome of HHM as reported in human patients (2). Nude mice with CAC-8 developed hypercalcemia, hypophosphatemia, increased serum 1,25-dihydroxycholecalciferol, increased urinary excretion of cAMP, and increased rates of bone formation and resorption without bone metastasis (2). Tumor extracts and conditioned tissue culture medium contained a factor which increased in vitro bone resorbing activity but was not suppressed by indomethacin, stimulated osteoclast hyperplasia and hyper trophy in neonatal mouse calvaria, and had transforming growth factor activity that was not dependent on the presence of epidermal growth factor (3, 4).

Humoral hypercalcemia of malignancy is an important clinical syndrome resulting from the secretion of unidentified substances by tumor cells which results in increased osteoclastic bone resorption, altered renal excretion of phosphorus and calcium, and increased nephrogenous cAMP (5). In HHM of human beings and laboratory animals there is increased total and fractional excretion of phosphorus and increased total and decreased fractional excretion of calcium (6, 7). These features also are a characteristic response to PTH excess (8). The effects on bone and kidney may result from one or more humoral factors released by tumor cells. Prostaglandins, PTH, TGFs, and PTH-like peptides have been suggested as the factors produced by tumor cells that result in the development of HHM (9). Most forms of HHM have been shown not to be the result of excessive secretion of PTH or prostaglandins (10). It has been hypothesized that HHM is due to a combined secretion of TGF and PTH-like peptides by the neoplastic cells (9). Transforming growth factors stimulate in vitro bone resorption and the TGF activity of the rat Leydig cell tumor and the Walker carcinosarcoma coelute on chromatography with bone resorbing activity (11, 12). Tumors associated with HHM in human beings and animals have been reported to contain factors which bind to PTH receptors in target cells and stimulate adenylate cyclase (13-15). It is not known whether the bone-resorbing activity and the adenylate cyclase-stimulating activity of tumors associated with HHM are the same or different proteins (15).

The tumor line CAC-8 contains in vitro bone-resorbing activity (3), TGF activity (4), and activity that stimulates adenylate cyclase in both bone and kidney. This triad of biological activities is typical of the few well-described laboratory animal models of HHM (8). PTH-like activity from a collection of human tumors associated with hypercalcemia correlated well with occurrence of HHM and could be used to classify the patients into "HHM" and "non-HHM" categories (15). In addition, the tumor line CAC-8 is a well-differentiated epithelial malignancy generally similar in type to the forms of cancer that produce HHM in human patients. The tumor doubling rate of CAC-8 in nude mice is approximately 14 days which allows time for morphological changes consistent with HHM to develop in nude mice.

The objectives of this investigation were to (a) determine whether hypercalcemic and normocalcemic canine adenocarcinomas contained activity capable of stimulating adenylate cyclase in ROS 17/2.8 (rat osteosarcoma) cells, UMR-106 (rat
osteosarcoma) cells, and OK (opossum kidney) cells; (d) evaluate the effects of forskolin and dexamethasone on adenylate cyclase stimulation; (e) determine whether adenylate cyclase stimulation could be inhibited by the PTH antagonist, $^{[14]}$Ne, $^{3}$Tyr$^{3}$BPTH (3-34); (d) compare the adenylate cyclase stimulation of CAC-8 extract to BPTH (1-34); and (e) determine the chemical stability and approximate molecular weight of the adenylate cyclase-stimulating activity in this animal model of HHM.

MATERIALS AND METHODS

Materials. Bovine PTH (1-34), forskolin, isoproterenol, dexamethasone, isobutylmethylxanthine, phenylmethlysulfonyl fluoride, pepstatin A, ATP, ADP, aAMP, adenosine, dithiothreitol, soybean trypsin inhibitor, and calcium- and magnesium-free Hanks' balanced medium Eagle's supplemented with 10% FBS, 2 mm L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and neomycin (100 µg/ml). The ROS cells are a cloned cell line from a rat osteosarcoma with a high level of AC responsiveness to PTH and prostaglandins (19). The ROS and UMR cells may represent different phases in the life cycle or different subpopulations of osteoblasts (18). The OK cells are an uncloned cell line derived from opossum kidney that have PTH-responsive adenylate cyclase (19).

Preparation of Tumor Extract. CAC-8 tumor tissue (0.5-2.0 g/mouse) was removed from hypercalcemic nude mice (Life Sciences, St. Petersburg, FL) and frozen at -70°C (13). Positive and negative control substances were added to result in specified concentrations to the wells for a treatment period of 5 min, and the reaction was stopped with the addition of 50 µl of 1.3 M trichloroacetic acid. The medium was transferred to plastic tubes and 50 µl of a carrier solution was added that contained 5 mM of ATP, ADP, AMP, CaAMP, adenosine, and dithiothreitol. The samples were frozen at -20°C overnight, thawed, and centrifuged for 10 min at 1200 × g. The supernatant was neutralized against 0.17 M KOH (25 µl), the cAMP was separated by the methods of Salomon (22) using serial Dowex X50 (Bio-Rad Laboratories, Richmond, CA) and alumina column chromatography, and the radiation was determined by scintillation spectrophotometry.

The effect of 4 canine tumor extracts (extracts 1 and 2 of CAC-8, CAC-22, and CAC-23) was determined on the AC activity of ROS cells. Bovine PTH (5-20 nM) was the positive control in each experiment. The effect of 2 h pretreatment of BPTH was evaluated on CAC-8- and BPTH-stimulated AC of ROS cells. A time response curve (1-20 min) was developed on the AC stimulation of UMR cells by CAC-8 extract. The dose response of AC stimulation by CAC-8 extract was determined using UMR and ROS cells. The effect of BPTH, isoproterenol (1 µM) (ß-adrenergic agonist), and CAC-8 extract (0.5 mg/ml) was compared on the AC stimulation of ROS, UMR, and OK cells. Forskolin (0.1 µM) was added directly to test substances or the 3 cell lines were incubated with medium supplemented with 30 nM forskolin for 48 h prior to the AC assay in order to determine the effects of forskolin or dexamethasone on the stimulation of AC in ROS, UMR, and OK cells by BPTH and CAC-8 extract.

The BPTH receptor antagonist, $^{[14]}$Ne, $^{3}$Tyr$^{3}$BPTH (3-34) amide was added to ROS, UMR, and OK cells in addition to BPTH (1-34) or CAC-8 extract to determine the dependence of AC stimulation on binding to the PTH receptor. Bovine PTH and CAC-8 extract were exposed to 60°C for 1 h, 100°C for 3 min, or trypsin-TCPK (50 µg/ml) for 1 h at 37°C to determine the effect of heat treatment and protease digestion on AC stimulation of UMR cells. The trypsin digestion was stopped by the addition of soybean trypsin inhibitor (100 µg/ml). The trypsin control solution contained both trypsin-TCPK and trypsin inhibitor at the same concentrations. CAC-8 extract was treated with dithiothreitol (0.062 M) for 1 h at 25°C and then extensively dialyzed against 0.17 M acetic acid. A paired batch of CAC-8 extract was treated identically without exposure to dithiothreitol.

Gel Chromatography. CAC-8 tumor extract (20 mg; 1 ml of sample) was dissolved in 1 n acetic acid, applied to a column (2.5 x 80 cm) of Fractogel TSK HW-50s (25-40 µm), and eluted with 1 n acetic acid at a flow rate of 2 ml/h. Fractions (1 ml) were collected, evaluated for absorbance of light at 280 nm, lyophilized, and stored at -20°C until assayed for AC-stimulating activity on UMR cells in the presence of forskolin (0.1 µM). Chromatographic molecular weight markers included bovine serum albumin (M, 67,000), chymotrypsinogen (M, 25,000), and insulin (M, 6,000).

Statistical Analysis. The AC stimulation assays were performed in triplicate. The data were expressed as cpm/well + SE. Data consisting of 3 or more groups were analyzed by analysis of variance and the New Multiple Range test (23). Data with 2 groups were evaluated with Student's t test. A significant difference was considered to exist when P < 0.05.
RESULTS

Three canine tumor extracts produced significant stimulation of AC in ROS cells (Table 1). The 2 batches of CAC-8 extract were not significantly different and produced approximately 47% the magnitude of stimulation of AC activity compared to BPTH. The extract from CAC-22 (a second anal sac adenocarcinoma from a hypercalcemic dog) significantly stimulated AC but was less active than the extracts from CAC-8 obtained by tumors grown in nude mice. Tumor extract designated CAC-23 (adenocarcinoma of anal sac from a normocalcemic dog) did not significantly stimulate AC activity as compared to CAC-8, CAC-22, and controls despite numerical doubling of AC activity (one-way analysis of variance). The protein content of CAC-8 extracts 1 and 2, CAC-22, and CAC-23 was 0.28, 0.29, 0.20, and 0.27 mg protein/mg dry extract, respectively. Pretreatment of ROS cells with BPTH for 2 h significantly reduced the response of AC to stimulation by both BPTH and CAC-8 extract by 45% (Table 2).

The time response curve (Fig. 1) of AC stimulation by CAC-8 extract demonstrates that cAMP accumulates in UMR cells in a linear pattern during the initial 10 min of incubation and then plateaus from 10–20 min. The point of median stimulation occurs at approximately 5 min. The dose response curve of AC cyclase stimulation in ROS cells (Fig. 2) by CAC-8 demonstrates a sigmoidal pattern with significant stimulation of AC at 0.01 mg of extract/well. In comparison, the UMR cells respond to CAC-8 extract with significant stimulation of AC at 0.035 mg CAC-8 extract/well (Fig. 3).

In Table 3 results are compared of AC activity in ROS, UMR, and OK cells after stimulation by BPTH, isoproterenol, and CAC-8 extract. The concentrations used for the 3 agonists were maximal for AC stimulation of the cell lines and were kept constant to permit comparisons between cell lines. Although the AC of ROS cells was stimulated by BPTH, isopro-
production of OK cells with or without forskolin, but the AC stimulation was increased significantly compared to controls only in the presence of forskolin. Forskolin, at 0.1 μM, did not stimulate the AC activity of the cell lines in the absence of another agonist. Preincubation of the cell lines for 2 days in medium with 30 nM dexamethasone significantly increased cAMP production by ROS cells in response to BPTH and CAC-8 extract. The AC stimulation of UMR and OK cells by BPTH was not potentiated by dexamethasone pretreatment, and cAMP production of UMR cells induced by CAC-8 extract was significantly reduced compared to control cells. Dexamethasone pretreatment of the cell lines did not alter cAMP production in the absence of an AC agonist.

The PTH receptor competitive antagonist [5,11Nle, 3Tyr]BPTH (3-34) amide significantly reduced AC stimulation of ROS, UMR, and OK cells by BPTH (1-34) and CAC-8 extract (Table 5). The [5,11Nle, 3Tyr]BPTH (3-34) amide did not stimulate AC activity of the 3 cell lines.

Heat treatment of CAC-8 extract (Table 6) demonstrated that the AC-stimulating activity of CAC-8 or BPTH in UMR terenol, and CAC-8 extract, the magnitude of AC stimulation by CAC-8 was significantly less than with BPTH or isoproterenol. Bovine PTH, isoproterenol, and CAC-8 extract stimulated the AC activity of UMR cells compared to controls, but the cells were significantly less responsive to isoproterenol. There was a numerical increase in cAMP production by OK cells in response to CAC-8, but when compared with BPTH (analysis of variance) it was not significantly different from controls. The UMR cells produced the greatest increase in AC activity compared to controls in response to CAC-8 extract (0.5 mg/ml) (Table 3). All 3 cell lines respond to a similar degree of BPTH, but there are significant differences in AC stimulation by CAC-8 extract and isoproterenol.

The effects of forskolin and dexamethasone on AC stimulation of ROS, UMR, and OK cells are presented in Table 4. The AC-stimulating assay was performed as described in "Materials and Methods." The data from each cell line were compared using one-way analysis of variance and New Multiple Range test.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ROS 17/2.8 (bone)</th>
<th>UMR-106 (bone)</th>
<th>OK (kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>237 ± 21°</td>
<td>230 ± 11°</td>
<td>538 ± 68°</td>
</tr>
<tr>
<td>BPTH (20 nM)</td>
<td>7219 ± 48°</td>
<td>5906 ± 263°</td>
<td>6593 ± 210°</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>8942 ± 126°</td>
<td>1394 ± 21°</td>
<td>485 ± 50°</td>
</tr>
<tr>
<td>CAC-8 (0.5 mg)</td>
<td>2639 ± 103°</td>
<td>5086 ± 507°</td>
<td>771 ± 72°</td>
</tr>
</tbody>
</table>

* Mean ± SE from triplicate wells of a representative experiment.
* Different from control, at P < 0.05.
* Different from no treatment, at P < 0.05.
* Different from BPTH and CAC-8, at P < 0.01.
* Different from forskolin, at P < 0.01.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No treatment</th>
<th>Forskolin (0.1 μM)</th>
<th>Dexamethasone (30 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS 17/2.8 (bone cells)</td>
<td>238 ± 20°</td>
<td>249 ± 15°</td>
<td>199 ± 13°</td>
</tr>
<tr>
<td>CAC-8 (0.05 mg)</td>
<td>797 ± 56°</td>
<td>1,349 ± 73°</td>
<td>1,690 ± 85°</td>
</tr>
<tr>
<td>BPTH (10 nm)</td>
<td>3,721 ± 34°</td>
<td>5,774 ± 91°</td>
<td>10,595 ± 684°</td>
</tr>
<tr>
<td>UMR-106 (bone cells)</td>
<td>295 ± 35°</td>
<td>310 ± 27°</td>
<td>479 ± 49°</td>
</tr>
<tr>
<td>CAC-8 (0.125 mg)</td>
<td>3,074 ± 16°</td>
<td>4,063 ± 91°</td>
<td>2,168 ± 214°</td>
</tr>
<tr>
<td>BPTH (10 nm)</td>
<td>5,918 ± 664°</td>
<td>7,947 ± 780°</td>
<td>6,567 ± 635°</td>
</tr>
<tr>
<td>OK (kidney cells)</td>
<td>449 ± 51°</td>
<td>275 ± 27°</td>
<td>497 ± 69°</td>
</tr>
<tr>
<td>CAC-8 (0.5 mg)</td>
<td>881 ± 93°</td>
<td>1,412 ± 126°</td>
<td>712 ± 112°</td>
</tr>
<tr>
<td>BPTH (10 nm)</td>
<td>3,672 ± 215°</td>
<td>9,597 ± 868°</td>
<td>3,130 ± 265°</td>
</tr>
</tbody>
</table>

* Mean ± SE from triplicate wells of a representative experiment.
* Different from control, at P < 0.05.
* Different from control, at P < 0.01.
* Different from control, at P < 0.001.
* Different from BPTH and CAC-8, at P < 0.01.
* Different from forskolin, at P < 0.01.
* Different from no treatment, at P < 0.05.

Table 5 Effect of f[5,11Nle, 3Tyr]BPTH (3-34) amide on stimulation of adenylate cyclase in ROS 17/2.8, UMR-106, and OK cells by BPTH and CAC-8 extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ROS 17/2.8 (bone)</th>
<th>UMR-106 (bone)</th>
<th>OK (kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>238 ± 20°</td>
<td>222 ± 20°</td>
<td>449 ± 51°</td>
</tr>
<tr>
<td>3-34 BPTH (1 μM)</td>
<td>205 ± 23°</td>
<td>385 ± 9°</td>
<td>190 ± 29°</td>
</tr>
<tr>
<td>BPTH (5 nM)</td>
<td>3721 ± 344°</td>
<td>7867 ± 867°</td>
<td>3672 ± 215°</td>
</tr>
<tr>
<td>BPTH + 3-34 BPTH (1 μM)</td>
<td>441 ± 51°</td>
<td>4050 ± 282°</td>
<td>1680 ± 237°</td>
</tr>
<tr>
<td>CAC-8 (0.05, 0.2, 0.5 mg)</td>
<td>797 ± 56°</td>
<td>4522 ± 587°</td>
<td>881 ± 93°</td>
</tr>
<tr>
<td>CAC-8 + 3-34 BPTH (1 μM)</td>
<td>186 ± 35°</td>
<td>846 ± 54°</td>
<td>230 ± 16°</td>
</tr>
</tbody>
</table>

* Mean ± SE from triplicate wells of a representative experiment.
* Different from control, at P < 0.01.
* Different from control, at P < 0.05.
* Different from BPTH, at P < 0.01.
* Different from forskolin, at P < 0.01.

Table 6 Effect of heat, trypsin, and dithiothreitol on the stimulation of adenylate cyclase in UMR-106 (bone) cells by CAC-8 extract and BPTH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No agent</th>
<th>CAC-8 (0.2 mg/ml)</th>
<th>BPTH (5 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>451 ± 46°</td>
<td>2175 ± 110°</td>
<td>5319 ± 44°</td>
</tr>
<tr>
<td>60 C, 1 h</td>
<td>2144 ± 69°</td>
<td>5694 ± 203°</td>
<td>4269 ± 439°</td>
</tr>
<tr>
<td>100 C, 3 min</td>
<td>2771 ± 27°</td>
<td>569 ± 66°</td>
<td>841 ± 96°</td>
</tr>
<tr>
<td>Trypsin (50 μg/ml)</td>
<td>538 ± 76°</td>
<td>569 ± 203°</td>
<td>4269 ± 439°</td>
</tr>
<tr>
<td>Control Dithiothreitol</td>
<td>2389 ± 340°</td>
<td>1444 ± 192°</td>
<td>5319 ± 449°</td>
</tr>
</tbody>
</table>

* Mean ± SE from triplicate wells.
* Different from control, at P < 0.01.
* Different from CAC-8 control, at P < 0.01.
weight was 34,000 (Fig. 4). Activity using UMR cells. The approximate relative molecular weight of clinical hypercalcemia. An anal sac adenocarcinoma which contained a protein factor which stimulated the AC of bone and kidney cells. The presence of AC-stimulating activity in the syndrome of HHM in nude mice as well as in the dog transplanted CAC-8 does include increased serum 1,25-dihydroxycholecalciferol, and increased bone formation (9). However, the syndrome of HHM which develops in nude mice with resultant PTH-like effects in tissues which are less responsive to the tumor products. The cell lines also varied in their AC response to the adrenergic agonist, isoproterenol, but in a different pattern compared to CAC-8 extract. ROS cells were the most responsive to isoproterenol, UMR cells were much less responsive, and OK cells did not respond to isoproterenol.

Although CAC-8 extract did not stimulate the AC complex of the 3 cell lines to the same degree as did BPTH, when the tumor extract was compared to BPTH by other physical and chemical means they resulted in parallel AC stimulation. Pre-treatment of ROS cells with BPTH for 2 h prior to exposure to CAC-8 extract or BPTH did result in desensitization of the AC stimulation similar to cultured chick kidney cells which undergo homologous desensitization to PTH after 1 h of preincubation with PTH (24). AC stimulation in response to CAC-8 extract and BPTH was enhanced in all 3 cell lines by the presence of forskolin (0.1 µM). This low concentration of forskolin alone did not stimulate cAMP production. Forskolin is a potent activator of the PTH-responsive adenylate cyclase system in a number of cell lines and can directly stimulate AC activity in cells at concentrations as low as 1.0 µM (25). The site of action of forskolin is unknown but may be the nucleoside regulatory unit of the AC catalytic system (25, 26). Preculture with dexamethasone enhanced AC stimulation by either CAC-8 extract or BPTH only in ROS cells (27). The mechanism of dexamethasone enhancement of AC in ROS 17/2.8 cells is unknown, but it has been reported that dexamethasone increased the guanine nucleotide regulatory protein of the AC catalytic system (28).

Specific fractions of CAC-8 extract obtained by gel exclusion chromatography demonstrated a single peak of AC-stimulating activity using UMR cells. The approximate relative molecular weight was 34,000 (Fig. 4).

DISCUSSION

The tumor line, CAC-8, which had been shown to produce the syndrome of HHM in nude mice as well as in the dog contained a protein factor which stimulated the AC of bone and kidney cells. The presence of AC-stimulating activity in canine adenocarcinomas correlated well with the development of clinical hypercalcemia. An anal sac adenocarcinoma which was taken from a dog with hypercalcemia contained AC-stimulating activity, but AC-stimulating activity was not detectable in an adenocarcinoma from a normocalcemic dog.

The cell lines utilized in this study, ROS 17/2.8, UMR-106, and OK cells, responded similarly to BPTH (1-34) but had a more varied response to CAC-8 tumor extract. The ROS cells responded to the lowest concentration of CAC-8 extract, but the UMR cells with equal concentrations of CAC-8 extract had the greatest increase in AC activity compared to controls. In response to equal concentrations of CAC-8 extract the cell lines produced approximately 50% (ROS cells), 80% (UMR cells), and 15% (OK cells) stimulation of AC activity compared to BPTH (1-34). This may indicate that the tumor-related factor which stimulated AC was not capable of interacting with different PTH receptors to the same degree as with native PTH. This observation is consistent with the hypothesis that more than one class of PTH receptors exist (9). The PTH-like factor(s) released by some tumors associated with HHM may interact with certain PTH receptors to a greater degree than with others. The bone and kidney alterations seen in human patients with hyperparathyroidism that are not present in patients with HHM include renal bicarbonate wasting, increased serum 1,25-dihydroxycholecalciferol, and increased bone formation (9). However, the syndrome of HHM which develops in nude mice with transplanted CAC-8 does include increased serum 1,25-dihydroxycholecalciferol and an increased bone formation rate (2). The reason for this difference compared to human patients may be due to an increased severity of HHM in the nude mouse model induced by a large tumor volume to body size ratio, and yet PTH-like effects in tissues which are less responsive to the tumor products. The cell lines also varied in their AC response to the adrenergic agonist, isoproterenol, but in a different pattern compared to CAC-8 extract. ROS cells were the most responsive to isoproterenol, UMR cells were much less responsive, and OK cells did not respond to isoproterenol.

Heat treatment of CAC-8 extract and BPTH at 60°C or 100°C did not reduce AC-stimulating activity. The cause of the small increase in AC-stimulating activity of CAC-8 extract after heating at 100°C is not known but may represent a direct enhancement of the AC-stimulating activity or removal of an inhibitory factor by denaturation. Bovine PTH and CAC-8 AC-stimulating activity were deactivated by exposure to trypsin indicating that both substances are proteins that were susceptible to trypsin-induced proteolysis. The AC-stimulating activity of CAC-8 extract was decreased significantly by reduction with dithiothreitol, suggesting that disulfide bonds are important structural components of the AC-stimulating protein.

Gel chromatography of CAC-8 extract demonstrated peak AC-stimulating activity at a relative molecular weight of 34,000. This is similar in size to AC-stimulating activities present in a human renal cell carcinoma, the rat Leydig cell tumor, and conditioned medium from human keratinocytes (29, 30). The PTH-like activity expressed by human keratinocytes may represent a similar factor that is released by squamous cell carc—

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Fig. 4. Gel exclusion chromatography of AC-stimulating activity in CAC-8 extract in UMR-106 (bone) cells. An acid-ethanol extract of adenocarcinoma (CAC-8) (20 mg, dissolved in 1 ml of 1 N acetic acid) was applied to a column of Fractogel TSK HW-50s (2.5 x 80 cm) and eluted with 1 N acetic acid at a flow rate of 2 ml/h. Fractions (1 ml) were collected, evaluated for absorbance of light at 280 nm, and assayed for AC-stimulating activity in the presence of forskolin (0.1 µM). BSA, bovine serum albumin (M, 67,000); C, chymotrypsinogen (M, 25,000); f, insulin (M, 6,000).
nomas associated with hypercalcemia. PTH-like peptides have been purified from a human squamous cell carcinoma and the rat Leydig tumor (H-500) (31). Peptides which were active in AC-stimulation assays had molecular weights of 9000 and 9500 by amino acid analysis. In addition, the rat Leydig cell tumor contained an active peptide of \( M_c 28,000 \). These findings suggest that the approximate molecular weights of AC-stimulating activities from gel exclusion chromatography experiments may be overestimated due to possible protein glycosylation and the lack of denaturing conditions. Two classes of AC-stimulating activity have been isolated from a murine squamous cell carcinoma model of HHM (32). Class I consisted of 3 closely related peaks observed in reverse phase high-performance liquid chromatography fractions that stimulated AC in disrupted and intact cells and were inhibited by \([\text{[\text{Ca}^2+]_{\text{m}}}}\text{[8]Nle, 34Tyr}]\)BPTH (3-34) amide. Class II was a single peak which stimulated AC only in disrupted cellular membranes and was not inhibited by \([\text{[\text{Ca}^2+]_{\text{m}}}}\text{[8]Nle, 34Tyr}]\)BPTH (3-34) amide. It was postulated that Class II AC-stimulating activity acts by a postreceptor mechanism and potentiates the activity of the Class I factor. The CAC-8 tumor-related peptide described in our study appears to more closely resemble Class I activity of the murine squamous cell carcinoma.

In summary the tumor line CAC-8 contains a protein factor(s) which stimulates the AC of bone and kidney cells by binding to the PTH receptor. The tumor-related activity was enhanced by forskolin and dexamethasone, was heat and acid stable, was inactivated by reduction, and had a relative molecular weight of approximately 34,000. Future purification and characterization of the AC-stimulating activity in CAC-8 will be useful in understanding the pathogenesis of HHM.

ACKNOWLEDGMENTS

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