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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

John Jerome Merendino, Jr.

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ABSTRACT

KERATINOCYTES PRODUCE A PTH-SENSITIVE

ADENYLATE CYCLASE-STIMULATING FACTOR

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Humoral hypercalcemia of malignancy (HHM) most frequently results from tumors of squamous epithelial origin. Studies suggest that the factor mediating HHM is distinct from PTH, but may act via the PTH receptor-coupled adenylate cyclase. This thesis addresses the hypothesis that non-malignant squamous epithelial cells produce a factor similar to that associated with HHM.

Ten cultures of normal human keratinocytes, each derived from a different neonatal foreskin epidermis, were grown to confluence on irradiated murine 3T3 fibroblasts or collagen. Keratinocyte-conditioned medium (KCM) from these cultures was tested in the PTH-sensitive rat osteosarcoma (ROS 17/2.8) assay, which responds to stimulation with PTH receptor agonists by producing [^{3}H]cAMP. KCM from each of the cultures demonstrated substantial adenylate cyclase-stimulating activity, generating from 4,400 to 31,280 cpm [^{3}H]cAMP/assay well, with basal being 1,400. This is equivalent to the stimulation produced by 1 x 10^{-10} to 8 x 10^{-10} M bovine parathyroid hormone (1-34) [bPTH-(1-34)]. Conditioned medium from four cultures of irradiated 3T3 cells and three cultures of dermal fibroblasts demonstrated no adenylate cyclase stimulation. Incubation with increasing concentrations of [Nle^{8,18}, Tyr^{34}]bPTH-(3-34)NH_2, a competitive inhibitor of PTH, progressively and completely prevented the KCM-induced [^{3}H]cAMP production. Incubation with a PTH antiserum inhibited the adenylate cyclase-stimulating activity of bPTH-(1-34), but not that of KCM. Concentrated KCM demonstrated dose-related production of cAMP in the PTH-sensitive renal cortical membrane adenylate cyclase assay. The active component of KCM is heat- and acid-stable, sensitive to trypsin and disulfide bond-reducing agents, and has a molecular weight of approximately 30,000 daltons as estimated by gel filtration, far greater than that of native parathyroid hormone.

These results indicate that keratinocytes produce a factor which is immunochemically and chromatographically distinct from PTH, but stimulates cAMP production via interaction with the PTH receptor. In every way tested thus far, the active component of KCM behaves identically to the adenylate cyclase-stimulating factor produced by tumors associated with HHM. This is the first report of such a factor to be obtained from non-neoplastic tissue. These studies suggest that HHM may result from the excessive secretion by tumors of a product produced by normal squamous epithelial cells.

TO MY PARENTS

For all the love, support, and
encouragement they have given me
throughout my life.

ACKNOWLEDGMENTS

With great joy I extend my thanks to those who helped me complete this work. The studies would not have been possible without Dr. Karl L. Insogna, who unhesitatingly furnished the material and expertise necessary to conduct the ROS assay, or Dr. Leonard M. Milstone, who provided tremendous support in the establishment and maintenance of keratinocyte cultures. I would also like to thank Dr. Mary Ann Mitnick, who conducted the PTH radioimmunoassay, and Ahmad Aminiafshar, who conducted the renal adenylate cyclase assay. Dr. Arthur E. Broadus shared his considerable experience in numerous helpful discussions during the course of my experiments. I owe my greatest debt, however, to my thesis advisor, Dr. Andrew F. Stewart, whose insight prompted these investigations, and whose enthusiastic support saw them to their completion.

CONTENTS

INTRODUCTION

Malignancy is the most common cause of hypercalcemia among hospitalized patients (1). Malignancy-associated hypercalcemia (MAHC) occurs in 10 to 20% of all cancer patients, and in up to 40% of patients with disseminated breast cancer, the neoplasm most often causing increased plasma Ca^{++} levels (2). Substantial evidence indicates that patients with MAHC fall into two groups based upon the pathophysiology of their increased extracellular Ca^{++} concentrations (3, 4). In the local osteolytic group, hypercalcemia results from direct destruction of bone by skeletal metastases. In the group having humoral hypercalcemia of malignancy (HHM), a blood-borne factor produced by tumor cells is thought to mediate bone resorption in the absence of skeletal metastases.

Epidemiologic data reveals that most tumors of a given histologic type tend to cause hypercalcemia via the same pathophysiologic mechanism. For example, in the vast majority of hypercalcemic patients with breast cancer or multiple myeloma, the increased plasma Ca^{++} results from bone resorption by metastatic lesions (3, 5). Similarly, certain tumors are most often associated with HHM. In the first large series of patients with hypercalcemia in the absence of bone metastases, Lafferty (6) reported that nearly 60% of cases resulted from squamous cell carcinomas, particularly those of the lung, and hypernephromas. Other investigators noted a high incidence of non-metastatic MAHC in patients with squamous carcinomas of the head and neck (7, 8) and esophagus (7, 9, 10). Stewart et al. (3) found that in nearly 80% of 50

unselected patients with cancer and increased plasma Ca^{++} levels, the hypercalcemia was humorally mediated. Most of these patients had squamous cell, renal or bladder carcinomas. Indeed, every large series of patients with HHM has reported the majority to have tumors of squamous cell epithelial origin, including those of the head and neck, lung, esophagus, cervix, vulva or skin, as well as renal, bladder or ovarian carcinomas (11, 12, 13, 14, 15).

Despite intense research, the humoral factor responsible for HHM remains unknown. As the prototypical bone-resorbing agent, PTH has long been regarded as a possible mediator. Indeed, patients with HHM share certain biochemical characteristics with those having primary hyperparathyroidism (1^{o} HPT). First, some patients with hypercalcemia and malignancy demonstrate apparent parathyroid hyperplasia (16), though most authors feel this is due to a loss of glandular fat associated with cachexia (17). In addition, patients with HHM or 1^{o} HPT both manifest hypophosphatemia as well as hypercalcemia. Parathyroid hormone is the only known substance to stimulate bone resorption and phosphorus excretion. This prompted Albright to propose that secretion of PTH by tumor tissue led to hypercalcemia in a patient with a renal carcinoma but only a single skeletal metastasis (18). In support of this notion, studies have revealed that patients with HHM or 1^{o} HPT also demonstrate 1)increased levels of nephrogenous cyclic 3',5' adenosine monophosphate (cAMP), and 2)reduced renal tubular phosphorus thresholds (3). The term "pseudohyperparathyroidism", first used by Fry (19) in the description of a patient with non-metastatic MAHC, illustrates the previous pervasiveness of the conviction that ectopic PTH secretion causes HHM.

Several authors have demonstrated the presence of immunoreactive parathyroid hormone (iPTH) in extracts or histologic sections of some tumors from patients with HHM (20, 21, 22, 23, 24, 25, 26), or in the culture medium of cells obtained from such tumors (27, 28, 29). However, other workers have failed to demonstrate iPTH in such tumors (14). Further, even in those studies which did report iPTH in HHM-tumors, the amount of hormone was generally 30 to 1000 times lower than that obtained from parathyroid adenomas (17). A review of these studies suggests that the use of relatively non-specific assay systems accounts for the frequency with which iPTH has been detected in HHM-associated tumor tissue (30).

Furthermore, despite the similarities between patients with HHM and 1^{o} HPT, these disease processes differ in important respects. For example, PTH increases bicarbonate excretion, so that patients with 1^{o} HPT often demonstrate a hyperchloremic acidosis. However, patients with pseudohyperparathyroidism most often have a hypochloremic alkalosis (6, 31). Additionally, recent work has shown that unlike patients with 1^{o} HPT, those with HHM manifest 1)markedly increased fractional Ca^{++} excretion, 2)decreased plasma levels of 1,25-dihydroxyvitamin D_3, and 3)low to undetectable plasma levels of iPTH (3, 32, 33, 34). Lastly, Simpson et al. (35), using molecular biological techniques, demonstrated that non-parathyroid tumors causing humorally-mediated hypercalcemia do not contain messenger RNA coding for parathyroid hormone. Since most of the studies which have implicated PTH in the pathogenesis of HHM utilized older, less specific assay systems, there is currently little evidence to support ectopic secretion of this hormone as the mechanism

underlying the development of hypercalcemia in cancer patients without skeletal metastases.

Various other substances have been implicated in the pathogenesis of HHM. For example, prostaglandin E_2 (PGE_2) stimulates cAMP production in bone-derived cell culture (36) and bone resorption in fetal rat calvaria (37). Infusion of PGE_2 into animals causes hypercalcemia (38). Tashjian and co-workers (39, 40) have shown that several animal tumors cause an HHM-like syndrome through the release of PGE_2. These investigators have demonstrated that indomethacin and hydrocortisone, inhibitors of prostaglandin synthesis, can prevent PGE_2 production by cultured tumor cells (41, 42) and correct hypercalcemia in tumor-bearing animals (43). Seyberth et al. (32) identified a group of patients with cancer, hypercalcemia and increased urinary levels of PGE-M, a PGE_2 metabolite, whose serum Ca^{++} concentration fell in response to treatment with prostaglandin synthesis inhibitors. These results are somewhat difficult to interpret in light of the finding that such agents inhibit the bone-resorbing effect of PTH in vitro (44) and prevent hypercalcemia in rats given PTH infusions (45). While prostaglandins may mediate bone resorption in certain instances, recent series have failed to establish a role for these compounds in the pathogenesis of hypercalcemia in large numbers of patients with HHM (46).

In addition to PTH and prostaglandins, vitamin D metabolites have been considered possible etiologic agents in HHM. Early reports suggested increased levels of certain vitamin D metabolites in patients with cancer and hypercalcemia (47), though subsequent studies

demonstrated no significant difference from control subjects (48). Still, macrophages are capable of 1α-hydroxylating 25-hydroxyvitamin D_3 to yield the maximally active hormone (49). Several authors have described patients with histiocytic lymphomas and hypercalcemia who displayed markedly increased plasma levels of 1,25-dihydroxyvitamin D_3 (30, 50). However, the vast majority of patients with HHM display strikingly low plasma 1,25-$(OH)_2$-D_3 levels (3). Thus, while vitamin D or its metabolites may mediate cancer-associated hypercalcemia in rare instances, there is no evidence to suggest that this mechanism operates in a large proportion of patients with HHM.

Currently, the two most active hypotheses hold that the substance underlying the development of HHM is either "growth factor-like" or "PTH-like". Epidermal growth factor (EGF) stimulates bone resorption *in vitro* either directly (51), or possibly by stimulating local production of PGE_2 (52). Transforming growth factors (TGFs) are substances produced by many solid tumors which stimulate cell growth and replication, and confer properties of the transformed phenotype onto target cells (53). Transforming growth factors share many characteristics with EGF. Some bind directly to the EGF receptor, while others require the presence of EGF in order to promote the expression of transformed properties in target cells (54, 55, 56). Ibbotson *et al.* (57) described the presence of a bone-resorbing factor having the properties of a TGF in medium conditioned by cells derived from an HHM-associated tumor in rats. D'Souza and co-workers (58) determined the active component of such medium to have a molecular weight of 30,000 daltons. This same group of investigators has recently presented

evidence that EGF receptor antiserum, though not PTH receptor antagonists, inhibit bone resorption by such conditioned medium (59).

Other investigators hold that while the bone-resorbing agent causing HHM differs from parathyroid hormone immunochemically, it may nonetheless share some functional characteristics with PTH. Indeed, a number of workers have demonstrated tumor-derived activity in various bioassays for parathyroid hormone. For example, Goltzman et al. (60) demonstrated that plasma from patients with HHM possessed increased PTH receptor-mediated glucose-6-phosphate dehydrogenase (G6PD) stimulating activity in an exquisitely sensitive cytochemical bioassay. Stewart et al. (61) found that tumor extracts from four of five patients with HHM stimulated the PTH-sensitive renal cortical membrane adenylate cyclase assay, as well as the G6PD cytochemical bioassay. These authors also demonstrated that tumor-derived activity was competitively inhibited by PTH receptor antagonists, but unaffected by PTH antiserum. In contrast, tumors derived from normocalcemic cancer patients, or patients with local osteolytic MAHC, demonstrated little or no PTH-like activity. Likewise, Rodan and co-workers (62), using a clonally derived line of rat osteosarcoma (ROS 17/2.8) cells which contain a PTH-sensitive adenylate cyclase, tested conditioned media from cultures of non-hypercalcemic and HHM-associated tumor cells. They found that conditioned medium from HHM-causing tumor lines stimulated cAMP production by ROS cells, as well as bone resorption in vitro, while medium from the normocalcemic lines did not. This adenylate cyclase-stimulating activity, too, was competitively inhibitable with PTH receptor antagonists. Recently, Gkonos et al., (63) using a

dimethylbenzanthracene-induced squamous cell carcinoma model of HHM in mice, demonstrated the adenylate cyclase-stimulating factor to have a molecular weight of 20,000 to 30,000 daltons.

Still other workers have exploited PTH-sensitive bioassays in molecular biological studies on cells from tumors causing HHM. Broadus et al. (64) injected polyadenylated RNA from HHM and control tumors into Xenopus oocytes, a method of preparing secretory translation products of mRNA derived from eukaryotic cells (65). Media from Xenopus oocytes injected with HHM-tumor mRNA stimulated the G6PD cytochemical bioassay in a competitively inhibitible fashion. Conditioned media from cultured oocytes injected with mRNA from control tumors demonstrated no cytochemical bioactivity. These studies all suggest that HHM-associated tumors produce a factor immunochemically distinct from PTH, but capable of stimulating cAMP formation and bone resorption via interaction with the PTH receptor.

In sum, current evidence indicates that HHM may be mediated by a protein product of tumor cells having a molecular weight of approximatley 30,000 daltons, which interacts with the PTH receptor-coupled adenylate cyclase complex in several assay systems, and may share some properties with tumor-derived transforming growth factors.

As stated previously, squamous epithelial carcinomas form the largest group of tumors causing HHM. This striking histologic uniformity raises the issue of whether normal squamous epithelial cells produce a factor similar to that secreted by their neoplastic counterparts. Under such

circumstances, HHM might result from the unregulated secretion by tumor cells of a normal cellular product. Until recently, studies of normal squamous epithelia were hampered by the inability to culture such cells readily. However, over the past several years methods for maintaining differentiating squamous epithelial cell cultures have been developed. The most widely studied such cells have been keratinocytes derived from human neonatal foreskin (66). This paper describes experiments in which conditioned medium from cultures of human neonatal keratinocytes was studied in the PTH-sensitive ROS and renal cortical membrane adenylate cyclase assays used in characterizing factors obtained from HHM-associated tumor tissue. The results indicate that cultured human keratinocytes produce a factor possessing physical and biological properties identical to those of the tumor cell product thought to mediate HHM.

METHODS

Cell Culture. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air. Except where indicated, keratinocytes were grown on a "feeder layer" of murine fibroblasts. BALB/c 3T3 fibroblasts CCL 92 (American Type Culture Collection, Rockville, MD) were grown to confluence in 150 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM; K.C. Biological, Lenexa, KA) supplemented with 10% bovine serum (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were exposed to 1200 rads of X-radiation, incubated with 10 ml 2.5 g/L trypsin in Hank's balanced salt solution (trypsin in HBSS; Gibco) at 37°C for 5 minutes, and dispersed by pipetting. 6×10^5 cells were then plated into 28 cm^2 dishes (Corning Glass Works, Corning, NY). Though unable to divide, these cells adhered to the culture flask and remained attached for 3-4 weeks, providing a suitable surface for keratinocyte proliferation.

Keratinocyte cultures were established according to the method of Rheinwald and Green (67), as modified by Milstone et al. (68). Human neonatal foreskins obtained at circumcision were placed in a complete medium (C-DMEM) consisting of DMEM with 20% fetal bovine serum (FBS; Gibco), 0.4 µg/ml hydrocortisone, 100 U/ml penicillin and 100 µg/ml streptomycin, and stored at 4°C until use, generally within 24 hours. Foreskins were washed twice with Ca^{++}-Mg^{++} free phosphate buffered saline (CMF PBS; Gibco) containing penicillin and streptomycin. The subcutaneous fat and deep reticular layer of the dermis were then dissected away with iris scissors, and the tissue was incubated with 10

ml of 2.5 g/L trypsin in HBSS at 4°C overnight. The following day, the epidermis was removed as a single sheet, placed in 10 ml of C-DMEM, and dispersed into single cells by vigorous pipetting. 2 to 4 x 10^5 cells in 5 ml C-DMEM were then plated into culture dishes containing irradiated 3T3 cells. One foreskin generally gave rise to 2-8 dishes on primary culture. Medium was changed two or three times per week. As the colonies of keratinocytes expanded, they displaced the 3T3 cells from the culture dish. The keratinocytes reached confluence in approximately 3 weeks, at which time they were subcultured by washing twice with CMF PBS and twice with 0.02% EDTA to remove residual 3T3 cells, incubating with 50 mg/L trypsin-0.2% EDTA in HBSS (Gibco) at 37°C for 15 minutes, dispersing into single cells, pelleting, resuspending in C-DMEM, and replating onto a feeder layer of 3T3 cells. On first passage, cells were generally split in a ratio of 1:8 or 1:10, yielding 20-60 28 cm^2 dishes per foreskin.

As indicated in Results, for specificity studies two keratinocyte cultures were established directly on culture dishes coated with collagen, according to the method of Liu and Karasek (69). All procedures were carried out as described above, except that the cells were maintained in a different complete medium (C-MCDB), consisting of Ham's MCDB 153 medium (K.C. Biologicals) containing 10% FBS, hydrocortisone, penicillin and streptomycin. C-MCDB was used for those cultures lacking feeder 3T3 cells since it is designed for the optimal clonal growth of keratinocytes. However, this medium contains a lower Ca^{++} concentration than C-DMEM, thus preventing the stratification of cultured keratinocytes (70).

Once the first-passage cells reached confluence, samples of keratinocyte-conditioned medium (KCM) were collected at each medium change, or under the special conditions described in Results. Harvested KCM was centrifuged at 500g for 5 minutes to remove desquamated cells, the supernatant was decanted and either assayed immediately or stored at -70°C until use.

Human fibroblasts were obtained from the dermis of neonatal foreskins following incubation with trypsin and separation of the epidermis. Dermal tissue was minced with iris scissors in 5 ml trypsin-HBSS and incubated at 37°C for 30 minutes. Repeated pipetting yielded single cells which were pelleted, resuspended in C-DMEM, and plated at 2×10^4 cells/cm^2. Medium was changed as with the keratinocyte cultures.

PTH-Sensitive Rat Osteosarcoma Cell Assay. Rat osteosarcoma (ROS) cells of the 17/2.8 strain, generously supplied by Dr. K. L. Insogna, were plated at 1×10^4 cells/cm^2 into 16 mm culture wells on 24 well plates (Costar, Cambridge, MA), and grown in 0.5 ml of F-12 medium (Flow Laboratories, McLean, VA) containing 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Medium was changed every 3 days. The cells became confluent 10-12 days after plating, and, as described below, were used in a modified form of the PTH-sensitive adenylate cyclase assay described by Majeska et al. (71) on the sixteenth day. This assay measures the production of intracellular cAMP by ROS cells in response to stimulation with PTH. These cells also generate cAMP in response to isoproterenol, though this is mediated via a separate receptor mechanism (72). Isoproterenol was therefore used in various experiments to detect

inhibition of the assay system not resulting from a specific interaction with the PTH receptor or its agonists.

The ROS assay was conducted at 37°C. Endogenous [^{3}H]ATP production was accomplished by incubating the cells with [^{3}H]adenine (ICN Biomedicals, Irvine, CA) for 2 hours. The incubation medium was then aspirated, and the wells were washed twice with 0.5 ml Ca^{++}-Mg^{++} free HBSS (Gibco). The cultures were then incubated with 0.1 ml/well F-12 medium containing 2% FBS and 0.2 mM isobutylmethylxanthine (Sigma, St. Louis, MO), a phosphodiesterase inhibitor. Following this, 0.1 ml of the sample to be assayed was added. C-DMEM served as the control agonist; bovine parathyroid hormone (1-34) (bPTH-(1-34); Beckman Bioproducts, Palo Alto, CA) was the standard. Dilutions of bPTH-(1-34), KCM, isoproterenol or other materials to be tested were prepared in C-DMEM. Cells were then incubated for 5 minutes in the case of bPTH-(1-34), or 10 minutes when other agonist materials were added (see Results). Each assay was performed in duplicate. The reaction was terminated with 20 µl of 1.2 M trichloroacetic acid. One hundred microliters of a carrier solution containing 5 mM each of cAMP, adenine, adenosine, ADP and ATP, and 40 µl of a recovery standard containing approximately 2000 cpm [^{32}P]cAMP (ICN) were then added. The reaction mixture was transferred to plastic tubes containing 0.88 ml H_2O, and frozen overnight at -20°C. The following day, samples were thawed, centrifuged at 3896 g in a Sorvall RC-5B refrigerated centrifuge (DuPont, Wilmington, DE) using an HS-4 rotor at 4500 rpm for 10 minutes. Supernatants were gently aspirated and neutralized with 4 N KOH in preparation for serial Dowex-alumina chromatography (see below).

PTH-Sensitive Renal Cortical Membrane Adenylate Cyclase Assay. Partially purified canine renal cortical membranes prepared by the method of Nissenson et al. (73) were kindly provided by Dr. A. F. Stewart. Each sample was assayed in duplicate. bPTH-(1-34) standards and KCM samples in volumes of 10 µl were added to 60 µl of a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 5.0 mM $MgCl_2$, 10mM KCl, 1 mM EGTA, 1 mg/ml bovine serum albumin (BSA), 0.125 mM ATP, 1.0 mM cAMP, 0.11 U/ml creatine kinase, 5 mM creatine phosphate, 2.0 mM dithiothreitol, 10 µM 5'-guanylyl imidodiphosphate (all reagents from Sigma) 100,000 cpm [α-^{32}P]ATP (Amersham, Arlington Heights, IL), and 60 µg renal cortical membrane protein. Reaction mixtures were incubated at 30°C for 30 minutes. The reaction was terminated by adding 0.1 ml of a "stopping solution" containing 2.0 mM ATP, 0.5 mM cAMP and 10,000 cpm [^{3}H]cAMP as a recovery standard, and heating to 100°C for 3 minutes. Reaction mixture volumes were then brought to 1.0 ml in preparation for serial chromatographic elution (61).

Chromatographic Separation of cAMP from ATP. [^{3}H]cAMP or [^{32}P]cAMP, produced in the ROS or renal adenylate cyclase assays, respectively, was separated from [^{3}H]ATP, [α-^{32}P]ATP and other radiolabelled nucleotides by the method of Salomon et al. (74). Ion-exchange chromatography columns prepared with 2.8 ml of a 50% slurry of Dowex AG-50W-X4 (200-400 mesh, hydrogen form; Bio-Rad Laboratories, Richmond, CA) were charged with 2 ml 1 N HCl and washed with 20 ml H_2O. Columns containing 600 mg Alumina WN-3 (Sigma) were washed with 10 ml 0.1 M immidazole-HCl, pH 7.4 (Sigma). Samples from either the ROS assay or renal adenylate cyclase assay were decanted onto the Dowex columns, which were then washed twice

with 3.0 ml H_2O. The first 3.0 ml eluate, containing primarily ATP, was discarded. The second eluate, containing the bulk of the cAMP, was passed over the alumina columns, to which the cyclic nucleotide adhered. An additional 1.0 ml H_2O wash, which served to elute residual cAMP from the Dowex, was passed sequentially over both columns and saved in a liquid scintillation vial. To this was added 5 ml of 0.1 M immidazole used to elute cAMP from the alumina column. This aqueous sample was mixed with 15 ml Hydrofluor counting cocktail (National Diagnostics, Somerville, NJ). Tritium and ^{32}P activities were then measured in a Minaxi Tri-Carb 4000 Series liquid scintillation counter (Packard Instrument Co., Downers Grove, IL), and corrected for recovery.

Protease Sensitivity Experiments. 100 µl of 2.5 g/L trypsin in HBSS was added to 1.0 ml samples of KCM, bPTH-(1-34) and isoproterenol prepared in C-DMEM. The samples were incubated at 27°C for 30 minutes, following which 20 µl of 25 g/L soybean trypsin inhibitor (Sigma) in 0.01M HAc-0.1% BSA was added. Samples were then incubated for an additional 10 minutes, and placed on ice until the time of assay.

Competitive Inhibition Studies. [Norleucine^{8,18}, Tyrosine^{34}] bovine PTH-(3-34) amide ([Nle^{8,18}, Tyr^{34}]bPTH-(3-34)NH_2; Bachem Fine Chemicals, Torrence, CA), a competitive inhibitor of PTH (75), was added to samples of bPTH-(1-34), KCM or isoproterenol in C-DMEM at the concentrations indicated in Results immediately prior to testing in the ROS assay. When assayed alone, the inhibitor was dissolved in C-DMEM.

PTH Antiserum Studies. The PTH-immunoreactivity of KCM was examined in two distinct studies. The first measured iPTH using an exclusively

carboxy-terminal radioimmunoassay utilizing G-6, a goat anti-human PTH antiserum (76), generously provided by Dr. L. Mallette, Houston, TX, and the terminal 48 amino acid fragment of bovine PTH [bPTH-(37-84)], courtesy of Dr. R. MacGregor, Kansas City, MO, labelled with ^{125}I, as the radioligand.

The second study examined the effects of antiserum pre-incubation on the ability of PTH, KCM and isoproterenol to stimulate cAMP production in the ROS assay. G-5, a goat anti-human PTH antiserum reacting with both amino- and carboxy-terminal portions of the PTH molecule (77), also provided by Dr. Mallette, was mixed with test substances at a final dilution of 1:1000. Samples were incubated at 27°C for 1 hour immediately prior to assay.

Gel Filtration Studies. A 5 ml sample of KCM was dialyzed against 4 L H_2O at 4°C overnight using Spectra-Por 3 dialysis tubing (Spectrum Medical Industries, Los Angleles, CA). The sample was then lyophylized, resuspended in 350 µl of 0.1 M HAc, and centrifuged at 500g for 5 minutes. Eighty microliters of the supernatant was saved for assay and protein determination; the remainder was subjected to gel filtration on Sephadex G-75 (Pharmacia, Piscataway, NJ) in a column with an effective bed volume of 30 cm^3, using 0.1 M HAc as the buffer. Two milliliter fractions were collected. 250 µl of each fraction was lyophilized to remove HAc, resuspended in the same volume of C-DMEM, and tested in the ROS assay.

Physical Characteristics. Samples of KCM were subjected to repeated freezing at -70°C and thawing at room temperature, snap-freezing in an

acetone-dry-ice bath, heating to 100°C for 10 minutes, lyophilization, 500 mM acetic acid, or incubation with 0.065 M dithiothreitol (Sigma) at 20°C for 20 hours prior to assay in the ROS system.

RESULTS

ROS Assay. Figure 1 illustrates a representative standard curve of the production of cAMP by ROS 17/2.8 cells in response to increasing doses of bPTH-(1-34). Accumulation of [^{3}H]cAMP varies almost linearly with the log of PTH concentration in the range of 5 x 10^{-11} to 10^{-9} M. Table 1 shows the adenylate cyclase-stimulating activity of KCM from 10 keratinocyte cultures, each obtained from a different foreskin, as well as that of conditioned medium from three cultures of dermal fibroblasts and 4 cultures of irradiated 3T3 cells. Eight of the keratinocyte cultures were grown on 3T3 cells in C-DMEM; the remaining 2 were grown on collagen in C-MCDB. The activity of each sample is given in terms of absolute [^{3}H]cAMP production as well as the equivalent concentration of bPTH-(1-34), using the standard curve generated in each assay.

One can readily see that each of the 10 samples of KCM possessed substantial adenylate cyclase-stimulating activity. For those keratinocytes grown on 3T3 cells, the production of [^{3}H]cAMP by KCM ranged from 8,170 to 31,280 cpm/well, with equivalent PTH concentrations being from 1.5 x 10^{-10} to 8.0 x 10^{-10} M. The adenylate cyclase-stimulating activity of KCM from keratinocytes grown on collagen was somewhat lower, with [^{3}H]cAMP production of 4,400 and 6,090 cpm/well, corresponding to PTH concentrations of 1.0 x 10^{-10} and 1.2 x 10^{-10}, respectively. This lower activity may have resulted from the fact that the lower Ca^{++} concentration of C-MCDB prevented these cells from stratifying (70). Cell numbers in these cultures were therefore several-fold lower, and the cells never underwent the differentiation

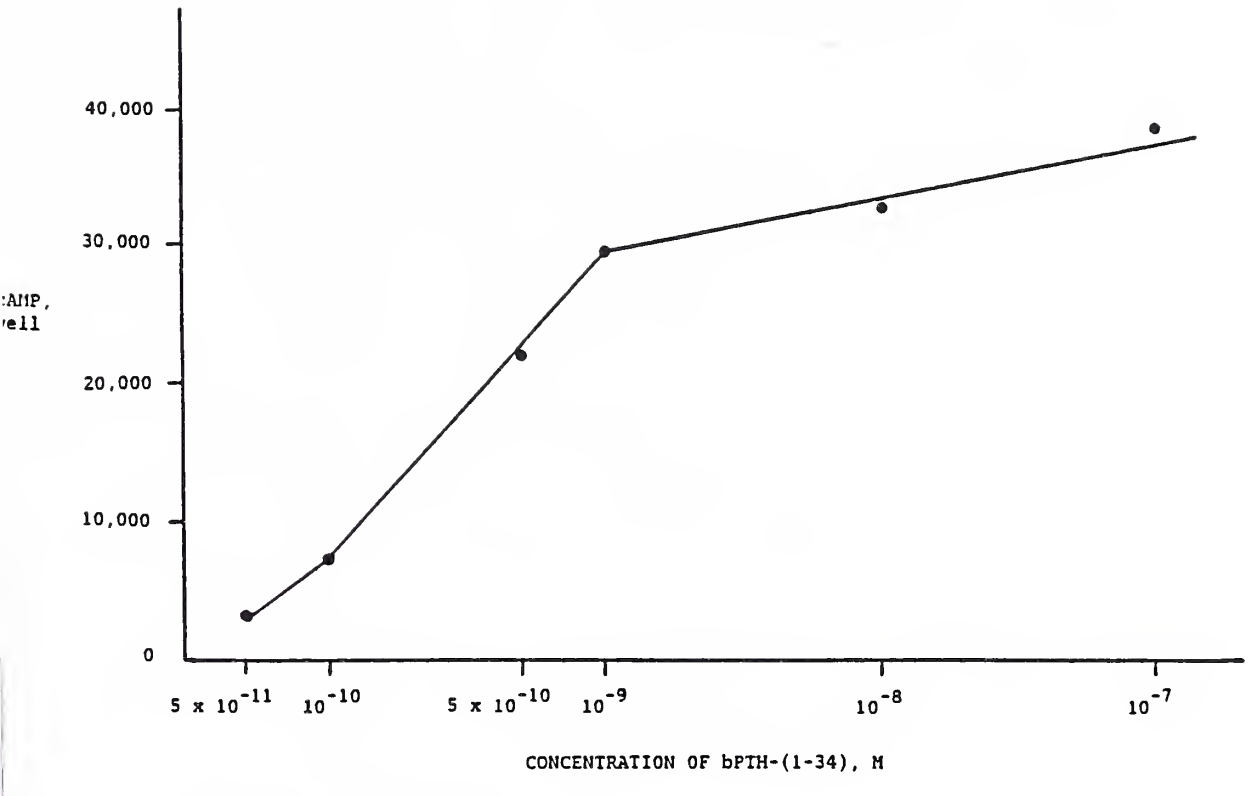

Figure 1. Dose-response curve of bPTH-(1-34) in C-DMEM. Production of [^{3}H]cAMP varies almost linearly with log[bPTH-(1-34)] over the range of 5 x 10^{-11} to 1 x 10^{-9} M. Basal (C-DMEM) in this assay was 430.

TABLE 1

Adenylate Cyclase-Stimulating Activity
of Conditioned Medium from Cultures of Keratinocytes,
Irradiated 3T3 Cells, and Dermal Fibroblasts

Medium Sample	[^{3}H]cAMP, cpm/well*	Equivalent bPTH-(1-34) Conc., 10^{-11} M#
Keratinocytes on 3T3 Cells		
KCM 1	11,660	16
KCM 2	8,170	16
KCM 3	31,280	80
KCM 4	12,600	19
KCM 5	11,010	16
KCM 6	10,520	15
KCM 7	17,150	29
KCM 8	9,400	15
Keratinocytes on Collagen		
KCM 9	6,090	12
KCM 10	4,400	10
3T3 Cells		
3T3 1	3,000	<5
3T3 2	1,440	<5
3T3 3	1,320	<5
3T3 4	1,020	<5
Dermal Fibroblasts		
DF 1	740	<5
DF 2	2,350	<5
DF 3	1,620	<5
Basal		
C-DMEM	1,415	<5

*[^{3}H]cAMP cpm/well represent the mean of values obtained for each sample in all assays tested.

#Equivalent bPTH-(1-34) concentration was obtained from a dose-response curve, such as that in Figure 1, generated in each assay. If a sample was tested in more than one assay, the mean value is given.

associated with progressive keratinization and desquamation. Nonetheless, these results indicate that keratinocytes grown without feeder fibroblasts produce ROS cell adenylate cyclase stimulating activity.

As stated previously, expanding colonies of keratinocytes displaced irradiated 3T3 cells from the surface of the culture dish. Confluent cultures, such as those from which KCM was obtained, were therefore largely devoid of feeder fibroblasts. However, since the cAMP-producing activity of those keratinocyte cultures grown on 3T3 fibroblasts was greater than that of cultures grown on collagen, conditioned medium from the murine cells themselves was studied for adenylate cyclase-stimulating capability. Several cultures of irradiated 3T3 cells were maintained in C-DMEM in the absence of keratinocytes. Medium conditioned by these cells was tested in the ROS assay, and the results are shown in Table 1. Conditioned medium from these cultures demonstrated no significant increase in [^{3}H]cAMP production above basal. While one sample did generate 3000 cpm/well [^{3}H]cAMP, in this particular assay the ROS cells responded to stimulation by PTH with greater than usual cAMP production. Therefore, this sample of 3T3 cell-conditioned medium possessed adenylate cyclase-stimulating activity lower than that of 5×10^{-11} M bPTH-(1-34).

Keratinocyte cultures may become contaminated with dermal fibroblasts owing to the imperfect separation of epidermis from dermis in the primary explant. Any keratinocyte culture demonstrating contamination with fibroblasts on phase-contrast microscopy was discarded. As an

additional control, however, conditioned medium from three fibroblast cultures, each derived from a different foreskin dermis, was tested in the ROS assay. As with the irradiated 3T3 cells, conditioned medium from these cultures demonstrated no significant increase in [^{3}H]cAMP production above basal. Collectively, these studies strongly suggest that the adenylate cyclase-stimulating activity of KCM was produced by keratinocytes, rather than the other cell types which may have been present in the cultures.

ROS Assay Incubation Time. Previous studies have demonstrated that various substances differ in the incubation time required to yield maximal [^{3}H]cAMP production in the ROS assay (62). PTH yields an optimal response after 5 minutes; hence, incubations with PTH were terminated with TCA after 5 minutes. Isoproterenol increases cAMP production for 10 minutes, so that incubations with this agonist were terminated after this period of time. Media conditioned by HHM-associated tumor cells also yields maximal [^{3}H]cAMP production at 10 minutes. In order to determine the time required to optimize the response of ROS cells to KCM, samples were incubated for 5, 10 and 15 minutes before the addition of TCA. Figure 2 demonstrates that [^{3}H]cAMP accumulates rapidly during the first 5 minutes, peaks by the 10 minute mark, and falls by 15 minutes. Therefore, all samples of KCM tested in the ROS assay were incubated for 10 minutes prior to acidifying the reaction medium.

Dose-Response Studies. In order to determine the response of the ROS system to varying concentrations of KCM, and to compare this behavior to

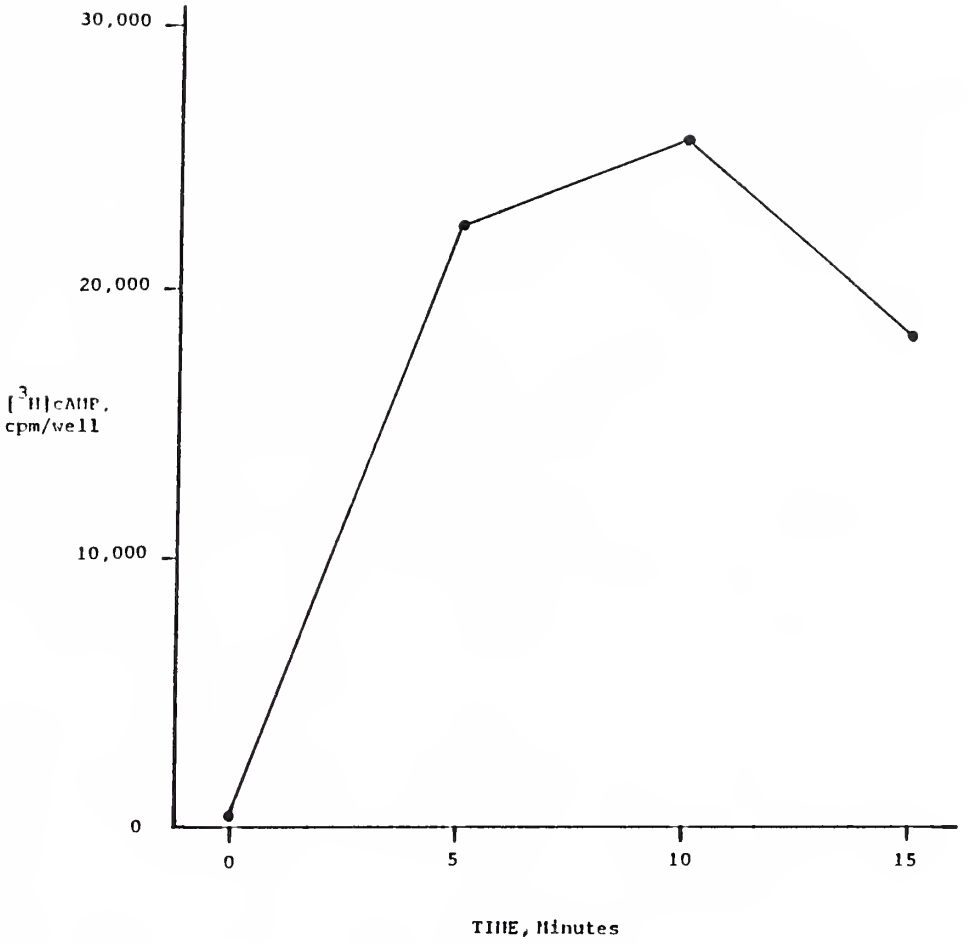

Figure 2. Time course of [^{3}H]cAMP production by ROS 17/2.8 cells in response to incubation with KCM. Cells were incubated with 0.1 ml F-12 medium containing 2% FBS and 0.2 mM isobutylmethylxanthine, as described in Methods. 0.1 ml of KCM was then added, and the cells were incubated for 5, 10 or 15 minutes further, following which 20 µl of 1.2 N TCA was added. As shown, the ROS cells demonstrated maximal [^{3}H]cAMP production following incubation with KCM for 10 minutes.

that obtained with other agonists, serial 2-fold dilutions of KCM in C-DMEM were tested in the ROS assay. Figure 3 illustrates the results, along with dose-response curves for PTH and isoproterenol. The curves for KCM and PTH demonstrate nearly identical slopes over the range of cAMP production corresponding to the linear portion of the PTH standard curve. In contrast, the isoproterenol curve has a slope nearly twice as great as that of KCM or PTH, and cAMP production rises to at least twice that produced by maximal PTH stimulation.

Time Course of Adenylate Cyclase-Stimulating Activity Appearance. In order to determine the time frame during which the adenylate cyclase-stimulating activity appeared in conditioned medium, samples of KCM taken from cultures at various times after medium change were tested in the ROS assay. The results of one such study are shown in Figure 4, upper curve. Samples were taken at 1, 2, 5, 10, 24, 48, 72 and 92 hours following medium change. The figure demonstrates that KCM had maximal cAMP-producing activity 48 hours after feeding. Repeat studies yielded virtually identical curves, indicating two days between medium changes to be optimal for collection of KCM.

Effect of FBS Concentration on Production of Adenylate Cyclase-Stimulating Activity. The effect of varying concentrations of FBS on the cAMP-producing activity of KCM was studied in anticipation of concentrating the responsible factor. Maintaining confluent cultures in medium with concentrations of FBS as low as 1% resulted in activity production identical to that seen with higher serum concentrations (data not shown). Cultures could be maintained in such low-serum medium for

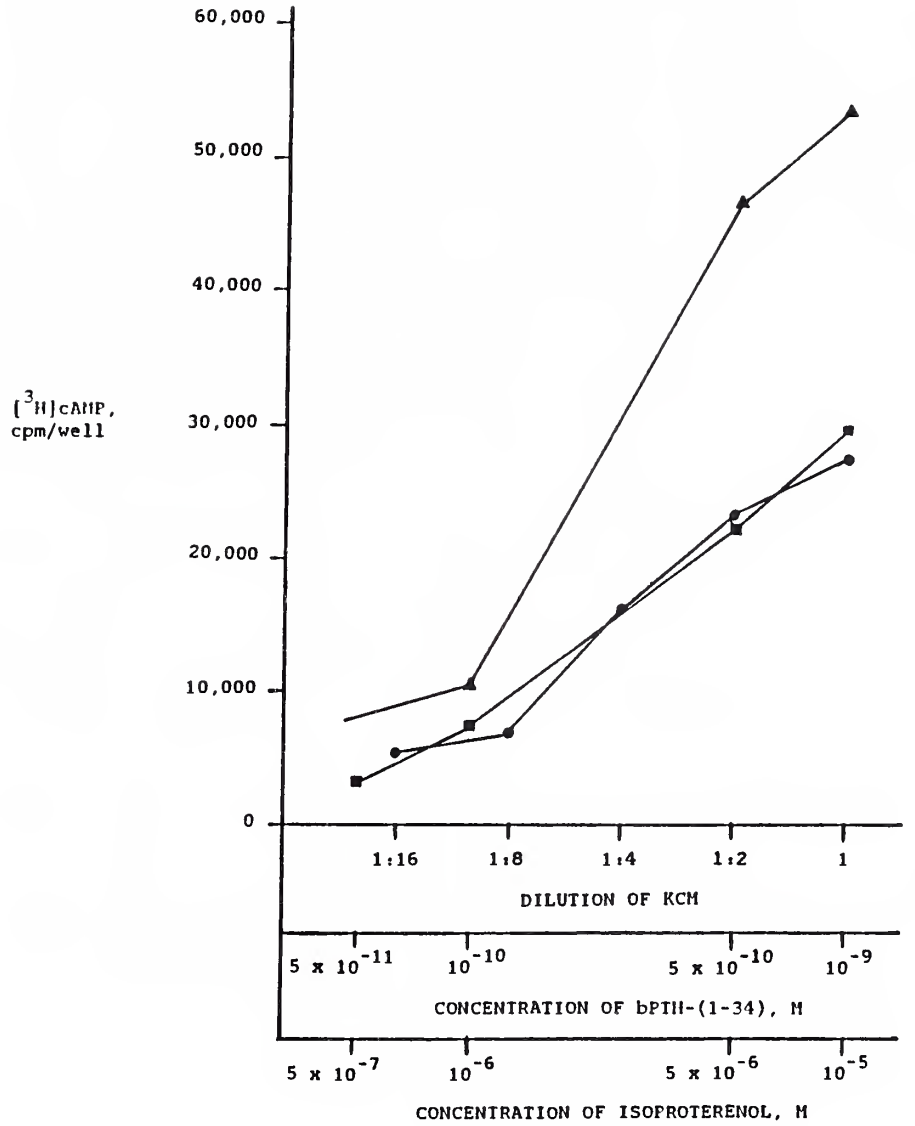

Figure 3. ROS assay dose-response curves for KCM (—●—), bPTH-(1-34) (—■—) and isoproterenol (—▲—). Note that KCM and PTH yield nearly identical curves, while that of isoproterenol has a slope twice as great.

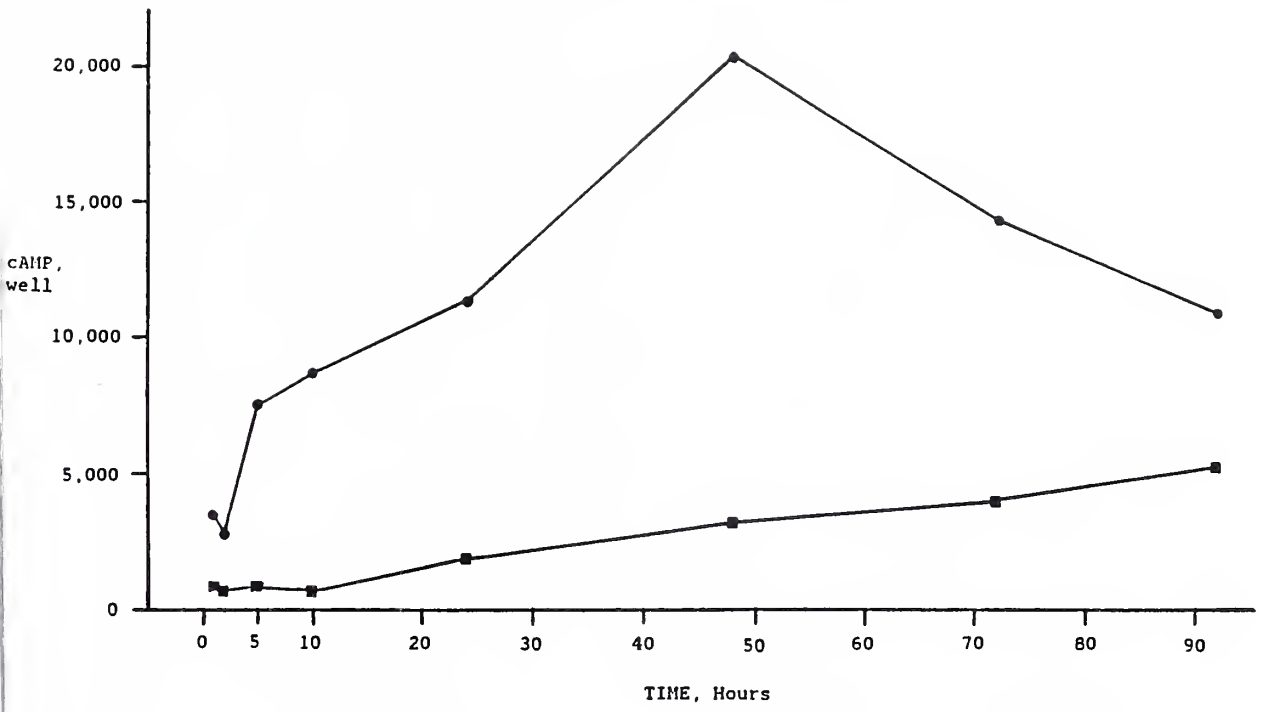

Figure 4. Time course of production of adenylate cyclase-stimulating activity by keratinocyte cultures. The upper curve (—●—) represents activity of KCM from a culture maintained in medium with 20% FBS (C-DMEM). The lower curve (—■—) represents activity of KCM from a culture maintained in serum-free medium. Basal in this assay was 430.

several feedings without significant loss of activity. However, cells washed with, and maintained in DMEM containing hydrocortisone, penicillin, and streptomycin, but lacking FBS, desquamated in large sheets after 2-3 medium changes. Further, even one such feeding with FBS-free DMEM resulted in a dramatic decrease in activity. Figure 4, lower curve, shows the time course of development of adenylate cyclase-stimulating activity by keratinocytes in the absence of FBS. Cells maintained in C-DMEM were washed three times and then fed with FBS-free DMEM, and the medium was sampled as before. The results differ from those obtained with C-DMEM in several respects. First, the maximal activity demonstrated by the serum-free KCM fell markedly below that which the same cells produced when fed with medium containing 20%, or even 1% serum. In addition, samples collected at 92 hours, rather than those taken at 48 hours, possessed the most activity. Finally, the activity of serum-free KCM shows an almost linear increase with time, rather than a rapid increase beginning soon after medium change.

Protease Sensitivity. In the first step toward characterization of the adenylate cyclase-stimulating activity produced by keratinocytes, the effect of trypsin digestion on KCM was studied in the ROS system. Figure 5 demonstrates that incubation with 250 mg/L Trypsin at 27°C for 30 minutes destroys virtually all of the cAMP-producing activity of both PTH and KCM, whereas trypsin incubation had relatively little effect on stimulation of adenylate cyclase by isoproterenol. The small inhibition of isoproterenol may have resulted from residual protease activity on the ROS cell membranes, despite the presence of trypsin inhibitor. Nonetheless, the assay system was still clearly capable of a vigorous

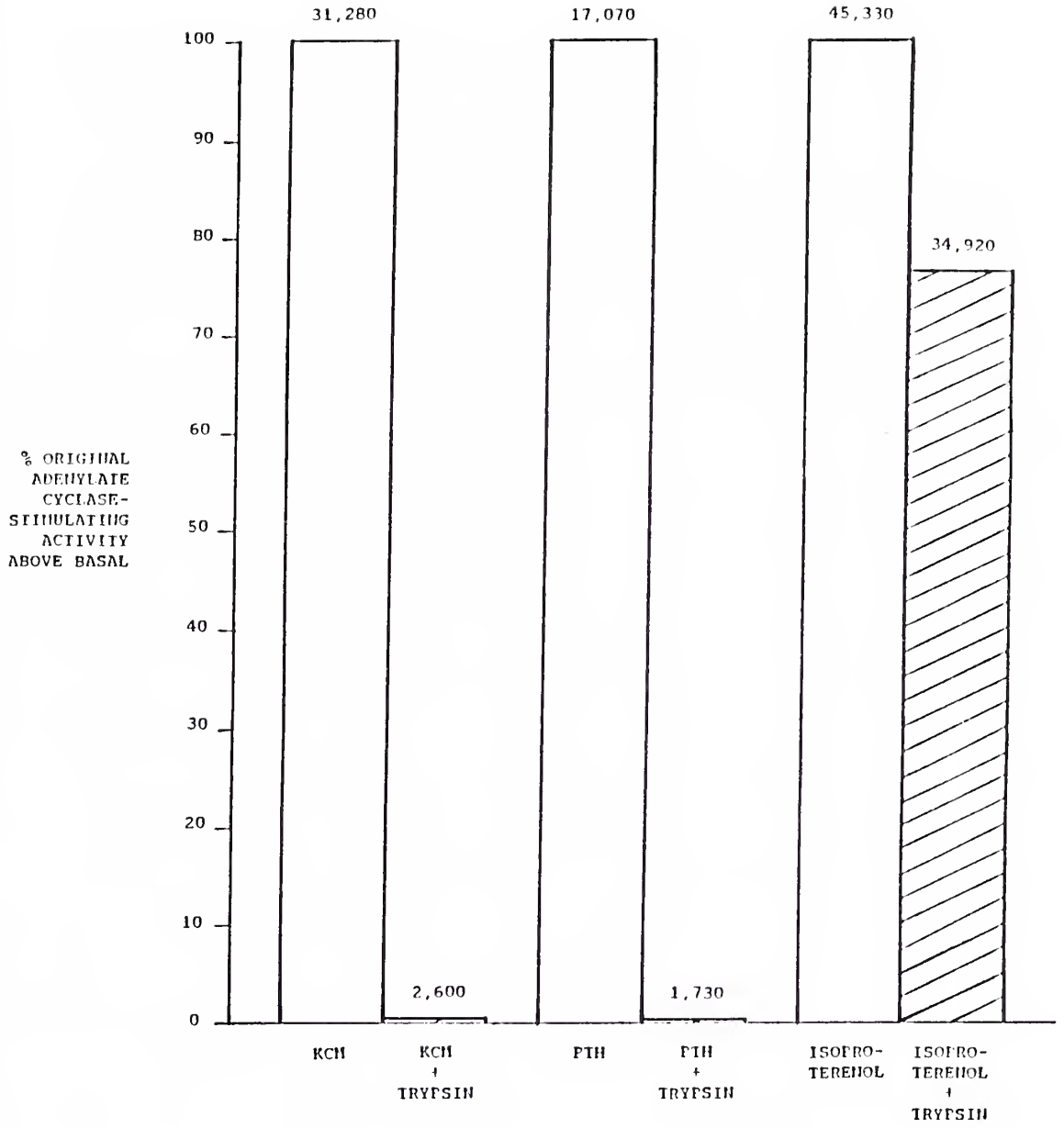

Figure 5. Effect of trypsin incubation on adenylate cyclase-stimulating activity of KCH, 2.5 x 10^{-10} M bPTH-(1-34) and 5 x 10^{-6} M isoproterenol in C-DMEM. Absolute [^{3}H]cAMP cpm/well are given above the bars in each instance. 1.0 ml samples were mixed with 0.1 ml of 2.5 g/L trypsin in HBSS and incubated at 27°C for 30 minutes. Following this, 20 µl of 25 g/L soybean trypsin inhibitor in 0.01 M HAc-0.1% BSA was added, the samples were incubated for an additional 10 minutes, and then placed on ice until the time of assay. Trypsin incubation completely eliminated the activity of KCH and PTH. The small inhibition of isoproterenol activity may have resulted from some residual protease activity on ROS cell membranes. The basal value in the assay for KCH and PTH was 2,340; for isoproterenol, basal was 950.

response in terms of cAMP production. These results indicate that the decreased activity seen following incubation of KCM with trypsin resulted from an effect of the protease on some constituent of the conditioned medium, strongly suggesting that the active factor in KCM is a protein or peptide susceptible to trypsin degradation.

Competitive Inhibition Studies. In order to assess the mechanism of stimulation of adenylate cyclase by conditioned medium, KCM was co-incubated with increasing concentrations of [$Nle^{8,18}$, Tyr^{34}]bPTH-(3-34)NH_2 prior to assay in the ROS system. In vitro, this compound binds to the PTH receptor with nearly the avidity of the native hormone, in that its inhibitory constant (K_i) virtually equals the affinity constant (K_m) for PTH (75, 78). However, the analogue is devoid of agonist properties except at high concentrations (75, 79, 80). Previous studies have shown that this compound inhibits the cAMP-producing activity of PTH in the ROS system, while it has no effect on the stimulation of adenylate cyclase by isoproterenol (62). Figure 6 illustrates the effect of increasing concentrations of this inhibitor on the production of [^{3}H]cAMP in response to incubation with KCM. Note that over the range of concentrations shown, the production of cAMP falls by over 80%, and that from 10^{-7} to 10^{-6} M, the decline is essentially linear. The results of this experiment were borne out in six replications: in every instance cAMP production in response to KCM progressively declined with increasing concentrations of inhibitor, and in each case activity fell to or slightly below that of the inhibitor alone. This was true even of KCM having the highest potency: [^{3}H]cAMP production in response to medium from culture 3 declined from 31,280 to

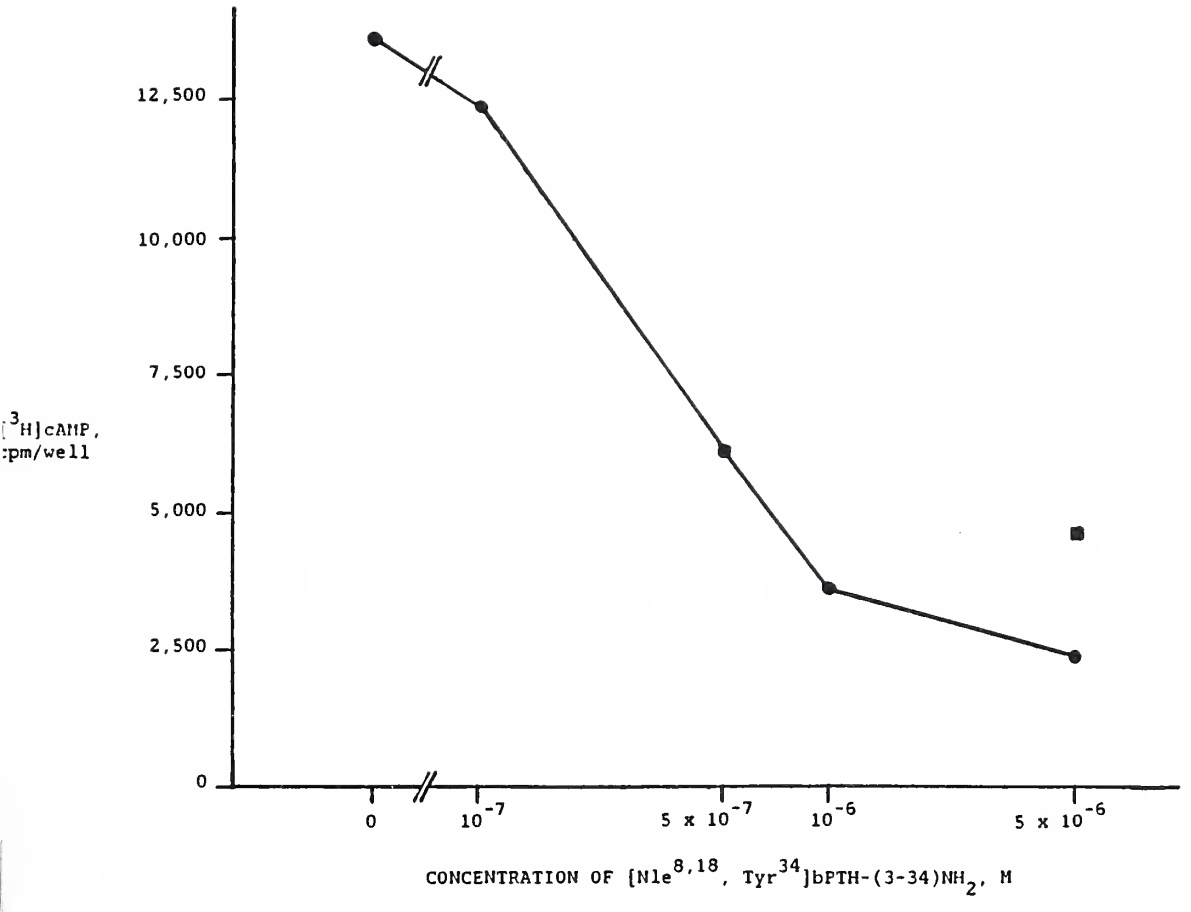

Figure 6. Effect of co-incubation with increasing concentrations of [$Nle^{8,18}$, Tyr^{34}]bPTH-(3-34)NH_2 on the adenylate cyclase-stimulating activity of KCM. Activity of KCM plus inhibitor is shown by the curve; activity of inhibitor alone at 5 x 10^{-6} M is shown by the square. Note that the production of [^{3}H]cAMP falls almost linearly with the log of inhibitor concentration over the range 1 x 10^{-7} to 1 x 10^{-6}. In seven such experiments with various samples of KCM, ROS assay activity declined progressively to or below the stimulation produced by the competitive inhibitor alone. Basal in this assay was 850.

3,621 cpm/well, with inhibitor alone at 5 x 10^{-6} M yielding 5,360 cpm/well.

PTH Antiserum Studies. The first study characterizing the PTH-immunoreactivity of KCM examined the concentration of iPTH in samples of conditioned medium from cultures of keratinocytes, dermal fibroblasts, and irradiated 3T3 cells. There was no significant difference between samples in the three groups, with each sample yielding less than 5% displacement of [^{125}I]bPTH-(37-84) on the standard curve. The maximum value obtained for any sample was 4 nlEq/ml, which corresponds to a bPTH-(1-34) concentration of less than 1 x 10^{-12} M, far below the limits of detectability of the ROS system.

The second study tested the effect of pre-incubating KCM, bPTH-(1-34) and isoproterenol with G-5 PTH-antiserum prior to testing in the ROS assay. Stewart et al. (61) have previously demonstrated that increasing concentrations of this antiserum block the stimulation of the renal cortical membrane adenylate cyclase by parathyroid gland extract, but not by extracts of tumors from patients with HHM. Figure 7 illustrates the results obtained in the ROS system. Incubation of 1 x 10^{-9} M bPTH-(1-34) with a 1:1000 dilution of G-5 at 27°C for 1 hour resulted in a fall of [^{3}H]cAMP-forming activity of more than 60%. In contrast, incubating G-5 at a 1:1000 dilution with KCM or isoproterenol resulted in modest declines of 13% and 16%, respectively, probably representing non-specific interference with the assay system by the antiserum. These results demonstrated little variability across a range of PTH concentrations and KCM activities. For example, when incubated with the

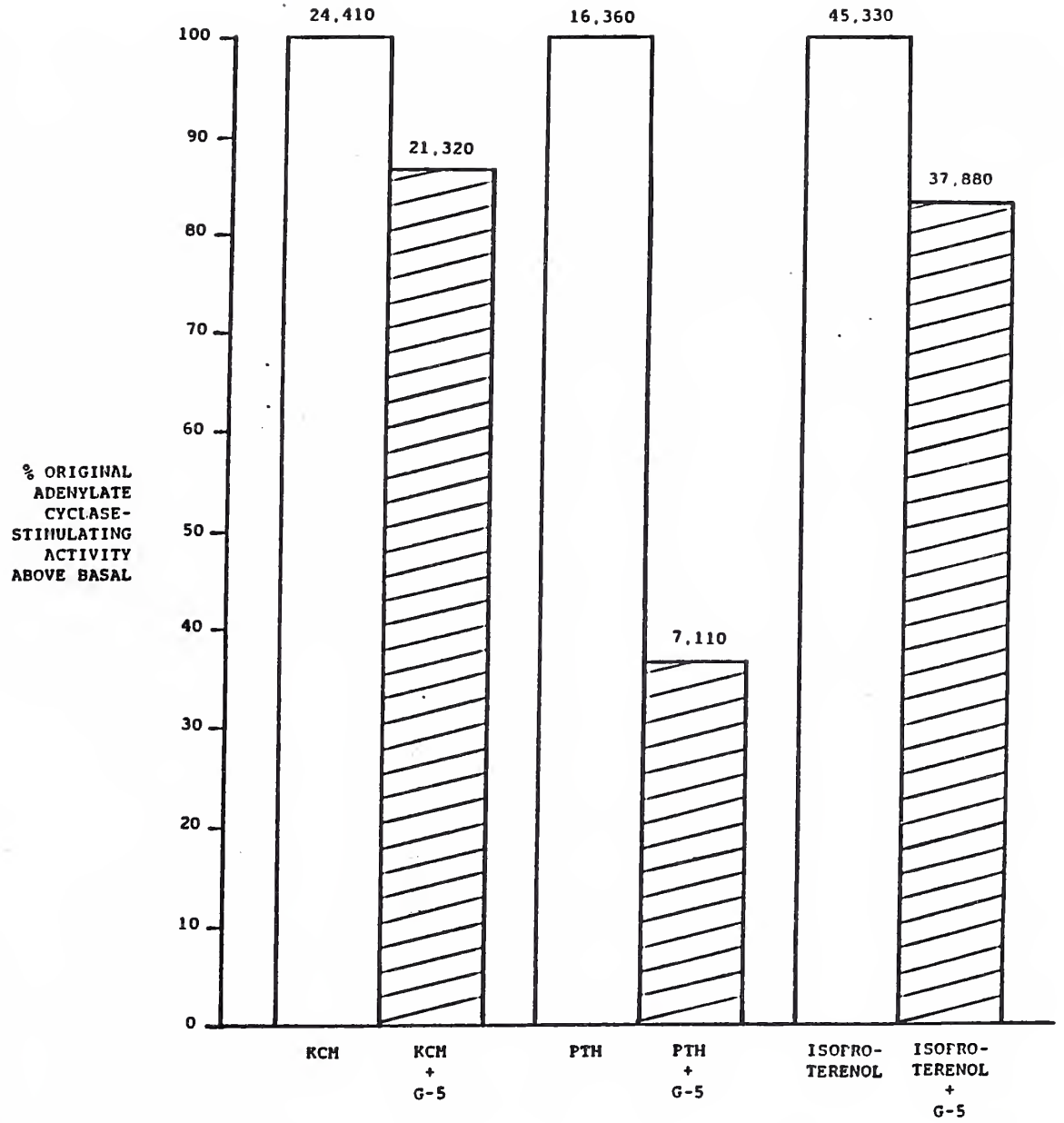

Figure 7. Effect of pre-incubation with G-5 PTH antiserum on the adenylate cyclase-stimulating activity of KCM, 1 x 10^{-9} M bPTH-(1-34) and 5 x 10^{-6} M isoproterenol in C-DMEM. Absolute [^{3}H]cAMP cpm/well are given above the bars in each instance. Samples were incubated with a 1:1000 dilution of G-5 antiserum at 27°C for 1 hour prior to assay. Pre-incubation with antiserum resulted in a 63% decrease in the cAMP-forming activity of PTH, but had little effect on that of KCM or isoproterenol. Basal for KCM was 1,260; for PTH, 1790; and for isoproterenol, 950.

same concentration of antiserum used in the above experiment, the activity of 2.5×10^{-10} M bPTH-(1-34) declined from 9,450 to 4,120 cpm/well, a fall of 65%, while the activity of KCM from culture 4 declined from 9,400 to 8,050 cpm/well, a drop of only 17%.

Gel Filtration and Physical Characteristics. Figure 8 shows the ROS cell adenylate cyclase-stimulating activity of fractions obtained from Sephadex G-75 gel filtration of concentrated KCM. As one can see, the cAMP-producing activity eluted in a discrete peak, with maximal activity in fraction 10 and substantial activities in fractions 9 and 11. This yields a preliminary estimate of approximately 30,000 for the molecular weight of the factor responsible for stimulation of adenylate cyclase in ROS cells. In contrast, native parathyroid hormone has a molecular weight of only 9,600.

Studies of the physical properties of the cAMP-producing factor in KCM indicate that repeated freeze-thaw cycles, snap freezing, and heating to 100°C for 10 minutes all had no effect on activity. Likewise, lyophilization, and treatment with 500 mM HAc did not decrease the activity of KCM. However, treatment with the disulfide bond-reducing agent dithiothreitol resulted in loss of adenylate cyclase-stimulating activity.

Renal Cortical Membrane Adenylate Cyclase-Stimulating Activity. Although the previous studies were all conducted in the ROS assay, this system is technically more cumbersome than the renal cortical membrane adenylate cyclase assay. However, the renal membrane assay is approximately 5- to 10-fold less sensitive to PTH than the ROS assay

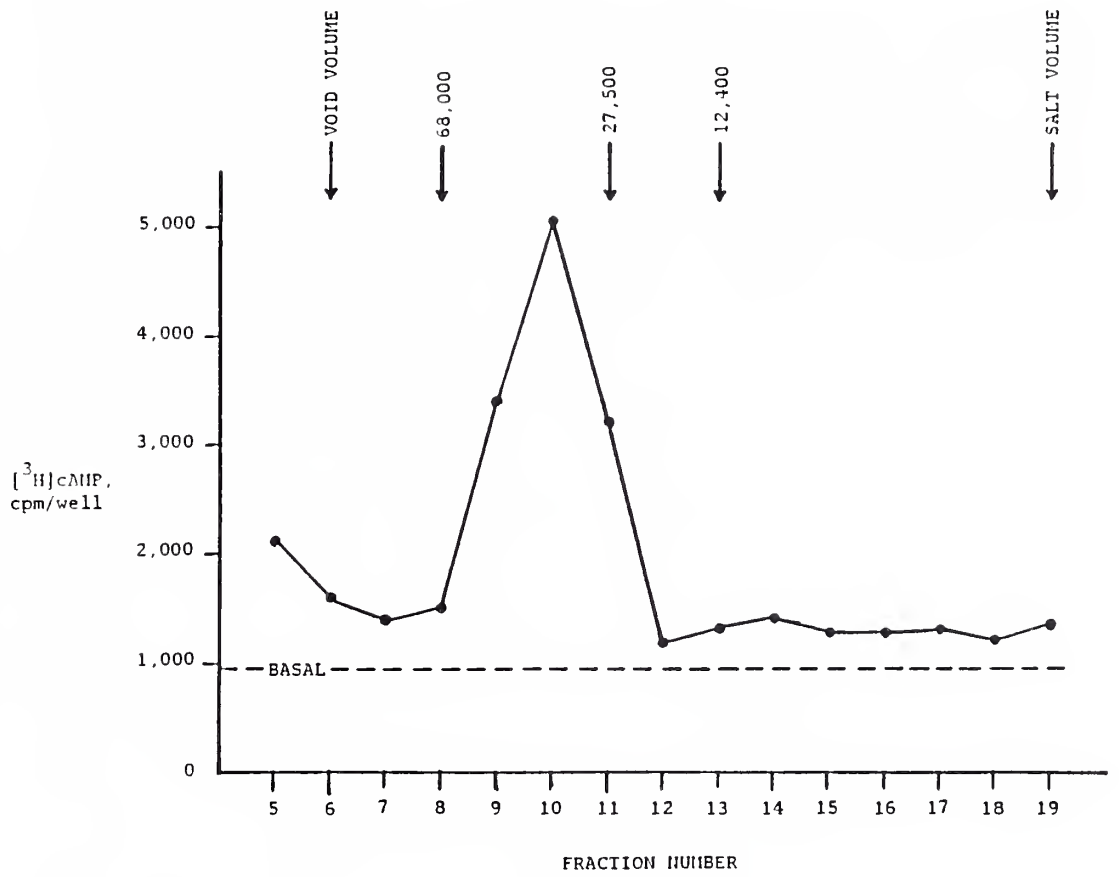

Figure 8. Sephadex G-75 gel filtration of concentrated KCM. A 5 ml sample of KCM was dialyzed against 4 L H_2O at 4°C overnight, lyophilized, and resuspended in 350 µl 0.1 M HAc. 270 µl of this solution was applied to a 30 cm^3 Sephadex G-75 column using 0.1 M HAc as the buffer. Two milliliter fractions were collected, from which 250 µl aliquots were lyophylized, resuspended in the same volume of C-DMEM, and tested in the ROS assay. A single discrete peak of adenylate cyclase-stimulating activity eluted with maximal activity in fraction 10, corresponding to a molecular weight of approximately 30,000 daltons. Column markers with molecular weights and elution fraction of peak activity were as follows: ferritin, >450,000, fraction 6 (void volume); BSA, 68,000, fraction 8; α-chymotrypsinogen, 27,500, fraction 11; cytochrome c, 12,400, fraction 13; tetracycline, 444, fraction 19 (salt volume).

(81), with 1 x 10^{-10} M bPTH-(1-34) being the lower limit of detectability. Maximal stimulation in this system, approximately 4-fold above basal, results from stimulation with 1 x 10^{-6} M bPTH-(1-34). When tested in the renal cyclase assay in unconcentrated form, samples of KCM possessing the greatest ROS activity yielded [^{32}P]cAMP production in the range of 400 cpm, only 1.5 times basal. Therefore, a sample of KCM was concentrated for use in renal adenylate cyclase assay. A 1.5 ml sample of KCM from a keratinocyte culture maintained in medium with 1% FBS was dialyzed against 4 L H_2O at 4°C for 5 hours, lyophylized, and resuspended in 35 µl 10 mM HAc. This sample, and a three-fold dilution in 10 mM HAc, were assayed as described in Methods. These samples yielded [^{32}P]cAMP production 2.28- and 1.70-fold above basal, respectively, corresponding to bPTH-(1-34) concentrations of 1.8 x 10^{-10} and 7.6 x 10^{-11} M. Although the responsiveness of the renal adenylate cyclase assay to KCM awaits further characterization, this preliminary result indicates that this system may be used in future study of the adenylate cyclase-stimulating factor produced by keratinocytes.

DISCUSSION

These studies were undertaken in order to test the hypothesis that squamous epithelial cells produce a factor similar to that obtained from tumors associated with HHM. The results indicate that cultured human keratinocytes produce a substance possessing physical and biochemical properties identical to those of the adenylate cyclase-stimulating and bone-resorbing factor obtained from HHM-associated tumor tissue. Factors produced by keratinocytes and tumors causing HHM both 1)stimulate cAMP formation in the ROS and renal cortical membrane assays (61, 62, 63); 2)stimulate the ROS assay optimally at 10 minutes (62); 3)are sensitive to incubation with trypsin (63); 4)differ immunoreactively from PTH (61); 5)are acid- and heat-stable (58); and 6)display an apparent molecular weight of approximately 30,000 daltons (58, 63). This is the first report of a factor having these characteristics to be obtained from non-neoplastic tissue. These studies raise the possibility that HHM may result from the unregulated secretion by tumor cells of a normal cellular product. Paraneoplastic syndromes frequently arise from the overzealous secretion by tumors of such factors (82). Indeed, some of the hormones whose regulation is now under study, such as ACTH and growth hormone, were first described as a result of the metabolic aberrancy caused by their excessive production in tumor tissue (83).

Though the experimental results strongly support the above conclusions, they raise important issues requiring further study. Perhaps the most difficult data to interpret are those regarding the

origin of the adenylate cyclase-stimulating activity of KCM. Conditioned medium from all ten keratinocyte cultures tested displayed cAMP-producing activity significantly above basal, while medium from cultures of irradiated 3T3 cells and human dermal fibroblasts displayed no such activity. However, the activity of KCM from those cultures grown on collagen was lower that of medium from cells grown on 3T3 fibroblasts. As indicated in Results, the different medium resulted in a smaller number of keratinocytes, and those that were present did not stratify. When grown under conditions promoting stratification, keratinocytes grown on collagen may demonstrate production of adenylate cyclase-stimulating activity as great as those grown on feeder fibroblasts. Further, even under these circumstances one would want to know the cell number and population dynamics in order to assess whether the process of keratinocyte differentiation affected activity (84). Finally, there is no evidence that decreased cell number or lack of differentiation alone accounts for the lower activity of these cultures. At present, one cannot exclude the possibility of an interaction between 3T3 cells and keratinocytes promoting production of a cAMP-forming factor.

While the experimental evidence indicates that neither 3T3 cells nor dermal fibroblasts generated the bioassay activity in KCM, other cell types, including melanocytes and Langerhans cells, reside within the epidermis, and thus may potentially contaminate keratinocyte cultures. Bringhurst et al. (85) describe a protease-sensitive bone-resorbing factor secreted by cultured tumor cells from a hypercalcemic patient with malignant melanoma. This raises the question of whether

melanocytes may have been the source of cAMP-producing activity in KCM. Selective culture of melanocytes requires conditions toxic to most other cell types (86), and these cells exist in very small numbers in keratinocyte cultures. More importantly, the factor produced by the melanoma line described above had an estimated molecular weight of 12,500, and, unlike the active component of KCM, failed to stimulate the PTH-sensitive adenylate cyclase in renal cortical membranes (85). Langerhans cells must be considered a potential source of hypercalcemia-inducing factor because they are a specialized type of tissue macrophage. As stated in the Introduction, macrophages are capable of producing 1,25-$(OH)_2$-D_3 (49), and patients with disorders of malignant macrophages (30, 50) or sarcoidosis (87) have hypercalcemia due to increased production of vitamin D. Vitamin D, however, stimulates neither the ROS nor renal cortical membrane assays, and shares none of the physical or chemical properties demonstrated by the active component of KCM. Furthermore, there exists no method for the selective culture of Langerhans cells, and, as with melanocytes, they constitute an extremely small proportion of the cells in keratinocyte cultures. These considerations argue strongly against either of these cell types as the source of adenylate cyclase-stimulating activity in KCM.

One must also recognize the limitations of the present study in analyzing the nature of the active factor in KCM. The fact that the dilution curves for bPTH-(1-34) and KCM have nearly identical slopes, while that of isoproterenol is far steeper, entices the conclusion that parathyroid hormone and keratinocyte conditioned medium stimulate

adenylate cyclase via the same mechanism. While the inhibition by [$Nle^{8,18}$, Tyr^{34}]bPTH-(3-34)NH_2 indicates that the active factor in KCM interacts with the PTH receptor, one cannot say that it does so in a manner identical to that of PTH. First, the studies did not fully characterize the response of the ROS system to stimulation with KCM. Unconcentrated medium from the most active keratinocyte culture yielded cAMP production near the level of maximal stimulation by PTH. As demonstrated by the studies with isoproterenol, however, the ROS cells are capable of much greater cAMP formation. Therefore, one can draw no conclusion regarding the maximal stimulation which can be obtained from the active component of KCM, and cannot speculate about the shape of its dose-response curve at higher concentrations. Likewise, since there is no information regarding the concentration of adenylate cyclase-stimulating factor in KCM, one can know nothing of its potency relative to PTH. Furthermore, the production of cAMP in response to stimulation of the PTH receptor results from a complex series of biochemical interactions (88). In contrast to binding studies, the assay system used here yields no information about the affinity of the PTH receptor for the agonist component of KCM.

Though the experimental evidence indicates a number of similarities between KCM- and HHM-derived adenylate cyclase-stimulating activities, one must take care in ascribing to the keratinocyte factor all the properties of the malignancy-associated bone-resorbing factor. Studies by Hermann-Erlee et al. (89) suggest that cAMP production and bone resorption may be mediated by PTH receptors with markedly differing affinities for the native hormone. Indeed, Rodan and Rodan (72) report

PTH receptor heterogeneity on ROS 17/2.8 cells, though binding to each type induced cAMP formation. These studies caution against attributing bone-resorbing activity to a factor merely on the basis of PTH receptor agonist activity in another system.

On the other hand, such receptor heterogeneity may provide insight into the question of why HHM resembles 1^{o} HPT in some respects but not others. Evidence from Strewler et al. (90) and others (91) indicates differing equivalent PTH potencies for the same preparation of HHM-factor in different assay systems. The ROS assay and fetal calvarial bone resorption assay appear to be 10- and 100-fold more sensitive to HHM-associated tumor extracts or conditioned medium than the renal cortical membrane assay relative to PTH. If HHM-causing tumors were to secrete a factor more potent in stimulating PTH-sensitive bone resorption and decreasing proximal tubular phosphorus reabsorption than in enhancing 1α-hydroxylation of 25-OH-D_3, a syndrome of hypercalcemia with reduced renal tubular phosphorus thresholds, increased nephrogenous cAMP and decreased plasma vitamin D levels might result.

At present, there is no information regarding a possible physiological function for the cAMP-producing factor in keratinocyte conditioned medium. Interestingly, dermal fibroblasts possess a PTH receptor-coupled adenylate cyclase (79, 80). Although the role of this complex in vivo is unknown, and there is no information regarding stimulation of fibroblast adenylate cyclase by KCM, the presence of such receptors raises the possibility that a PTH receptor agonist serves a

regulatory function in human skin. One might postulate that the PTH-like factor produced by keratinocytes is the in vivo agonist of these receptors. Indeed, there are numerous examples of autocrine and paracrine factors affecting the growth and differentiation of dermal and epidermal cells (92).

Alternatively, the keratinocyte-derived factor may have some systemic physiological function. Little is known of the part played by skin in Ca^{++} metabolism except that it is the source of photochemically generated cholecalciferol (93). However, various dermatological conditions other than squamous carcinoma of the skin are associated with derangements in Ca^{++} balance. For example, psoriasis vulgaris (94, 95), pustular psoriasis of von Zumbusch (96, 97, 98, 99), and impetigo herpetiformis (100, 101, 102) occur in the context of hypocalcemia. Most often, the low calcium results from decreased albumin levels (98, 103, 104, 105, 106). However, in some instances decreases in ionized Ca^{++} have occurred as a result of hypoparathyroidism or malabsorption (94, 95, 97, 99, 102). In some such cases, treatment with vitamin D analogues or Ca^{++} infusions has been successful (107). These dermatological syndromes all involve keratinocyte hyperplasia. One might speculate whether the increased rate of keratinocyte proliferation generates larger quantities of adenylate cyclase-stimulating activity, leading to enhanced bone resorption with amelioration of the hypocalcemia.

Definitive information regarding the nature of the factor responsible for the adenylate cyclase-stimulating activity produced by keratinocytes

must await purification in sufficient quantities to permit analysis of the structure by conventional chemical methods. The first step in producing such purified material would be to increase the specific activity of the protein in KCM by lowering the content of FBS. As indicated in Results, keratinocytes can be maintained for several feedings in low-serum medium without significantly decreased factor production. Although the total absence of FBS markedly lowered cAMP-producing activity, this was using media not supplemented with specific substances which enhance keratinocyte growth. Maciag and co-workers (108, 109) have developed a method for maintaining keratinocytes in a serum-free medium. If keratinocytes maintained in such a fashion were to demonstrate significant production of adenylate cyclase-stimulating activity, this would greatly facilitate purification of the responsible factor. Further studies are necessary in order to assess the feasibility of larger-scale factor production in low-serum or serum-free medium.

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