PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 438–442 (1986)

Forskolin-Induced Bone Resorption in Neonatal Mouse Calvaria *in Vitro* (42278)

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Abstract. The role of cyclic adenosine 3',5'-monophosphate (cAMP) in inducing bone resorption was studied in neonatal mouse calvaria *in vitro*. Forskolin, a stimulator of adenylate cyclase, increased the medium calcium concentration at 96 hr of incubation, indicating enhanced bone resorption. Bone resorption was observed between 1×10^{-4} and 1×10^{-6} *M* forskolin; the maximal effect was at 1×10^{-5} *M* and there was no effect at 1×10^{-7} *M*. Lactic acid release was increased during the 96 hr of incubation in proportion to the calcium release in the media. The bone acid phosphatase activity was increased and the alkaline phosphatase activity was decreased. Bone carbonic anhydrase activity was increased more than twofold. Forskolin-induced bone resorption was significantly but incompletely inhibited by 10^{-4} *M* acetazolamide, a carbonic acid anhydrase inhibitor. These findings support the concept that carbonic anhydrase plays a significant role in bone resorption.

Parathyroid hormone (PTH) is a major physiological regulator of bone resorption. The earliest apparent effect of PTH is a stimulation of adenylate cyclase and an increase in the cyclic adenosine 3',5'-monophosphate (cAMP) content of the bone. A marked increase in cAMP is detected within 1 min after adding PTH to *in vitro* cultures of fetal rat calvaria; the maximal effect is observed within 5 min (1). Other effects of PTH are detected only subsequently to this event. Exposure of skeletal tissue *in vitro* to the cAMP analog dibutyryl cAMP results in biological effects that parallel stimulation by PTH (2). Administration of dibutyryl cAMP to parathyroidectomized animals elevates the serum calcium levels (3), an effect which has been interpreted as reflecting bone resorption. It is generally accepted that cAMP may serve as an intracellular messenger for the osteolytic response to PTH (1, 3).

Forskolin is a potent activator of adenylate cyclase in many tissues and cells (4). It is assumed that forskolin, which is lipophilic in nature, is able to pass through cell membranes with relative ease and stimulate adenylate cyclase intracellularly. This property of forskolin is in contrast to the stimulatory effects of other agents. On the one hand, many peptide hormones, such as PTH, act extracellularly through a receptor coupled to adenylate cyclase. On the other hand, certain agents, such as fluoride, are able to stimulate the enzyme directly, but only in broken cell preparations, as penetration of fluoride through the cell wall is restricted.

The present study was undertaken to further our understanding of the role of cAMP in bone resorption and the pathway by which the latter effect is mediated. The effects of forskolin on calcium release, lactic acid production, and on three bone enzymes (acid and alkaline phosphatases and carbonic anhydrase) were examined in neonatal mouse calvarial cultures *in vitro*. A preliminary report of this work has appeared elsewhere (5).

Materials and Methods. *Media and chemicals.* Media and antibiotics were purchased from either GIBCO Laboratories (Grand Island, N.Y.) or K.C. Biological (Lenexa, Kans.). Chemicals were obtained as follows: bovine serum albumin (Fraction V) from Sigma Chemical Company (St. Louis, Mo.); forskolin from Calbiochem (La Jolla, Calif.); and acetazolamide from Lederle Laboratories (Pearl River, N.Y.). Forskolin was dissolved in a vehicle containing dimethyl sulfoxide and ethanol (1:1). All water used was glass-distilled.

Bone culture. Calvaria were removed from 5- to 6-day-old mice aseptically and cut along the sagittal suture into two halves. One half of the calvarium was used for control (vehicle only) and the other half for treatment (forskolin). The right and left half of each calvarium were alternated between control and treatment groups. Each half calvarium was cultured in a 35-mm sterile plastic petri dish (Cat. No.

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1008, Falcon, Oxnard, Calif.) containing 1.5 ml of Dulbecco's modified eagle medium (high glucose) supplemented with 0.25% bovine serum albumin (Fraction V). Forskolin or its vehicle was added to the medium prior to dispensing the latter into the culture dish. The highest concentration of forskolin used (10^{-4} *M*) contained 1% of the vehicle. At 10^{-5}, 10^{-6}, and 10^{-7} *M* forskolin the concentration of vehicle was 0.1, 0.01, and 0.001%, respectively. Control and treated media always contained the same concentration of vehicle for each concentration of forskolin used. Culture dishes were incubated in 5% CO_2/95% air at 37°C in a water-saturated atmosphere. All cultures were maintained for 96 hr; the media were changed at 48 hr.

Assessment of bone resorption. Bone resorption was assessed by changes in the medium calcium concentration at the end of the 96-hr incubation period. Calcium concentrations were determined using a Technicon AutoAnalyzer II system fitted with a calcium manifold.

Estimation of lactic acid. Lactic acid concentration in the medium was estimated with a diagnostic kit obtained from Sigma Chemical Company. The lactic acid was converted to pyruvic acid by lactate dehydrogenase resulting in the reduction of an equivalent amount of NAD. The increase in absorbance at 340 nm is proportional to the lactic acid concentration and was detected with a Beckman Model 34 spectrophotometer.

Preparation of bone homogenates. At the end of the culture period, the calvaria were removed from the media and gently blotted. After washing in ice-cold 0.25 *M* sucrose solution they were reblotted and placed in ice-cold 0.25 *M* sucrose solution (5 half calvaria per 1.5 ml sucrose solution). The bones were homogenized with a Polytron PCU-2 homogenizer using three 20-sec bursts at a speed setting of 5. Each homogenate was centrifuged at 600*g* for 10 min. The supernatant was saved and the pellet was suspended in 0.375 ml of an ice-cold solution containing 0.25 *M* sucrose and 0.5 *M* KCl. The suspension was centrifuged at 600*g* for 10 min. The resulting supernatant was combined with that previously obtained and the final pellet was discarded.

Protein determination. Protein concentrations were determined using the method of Lowry *et al.* (6) as modified by Oyama and Eagle (7). Bovine serum albumin was used as the protein standard.

Enzyme assay. Acid and alkaline phosphatase assays were patterned after the method of Lieberherr *et al.* (8). Acid phosphatase was assayed in pH 4.8, 0.05 *M* acetic acid/Na acetate buffer containing 0.1% Triton X-100, with 5 m*M* *p*-nitrophenyl phosphate as substrate. Assays were carried out at 37°C for 30 min and were terminated by the addition of 2.5 vol of 0.1 *N* NaOH. Alkaline phosphatase was assayed in pH 9.5, 0.1 *M* triethanolamine/HCl buffer containing 0.05 *M* $MgCl_2$ with 15 m*M* *p*-nitrophenyl phosphate as substrate. Assays were carried out at 37°C for 15 min and were terminated by the addition of an equal volume of 0.1 *N* NaOH followed by centrifugation at 500*g* for 10 min. Both acid and alkaline phosphatase assays were read at 405 nm in a Beckman Model 34 spectrophotometer using a molar extinction coefficient (E_M) of 1.6×10^4 $M^{-1} \cdot cm^{-1}$ for *p*-nitrophenolate. Assays containing boiled homogenates were used to correct for nonenzymatic product formation.

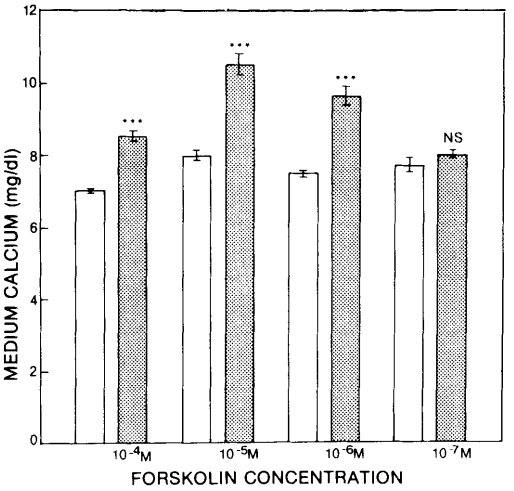

FIG. 1. Effect of forskolin on calcium release from neonatal mouse half calvaria *in vitro*. Half calvaria were cultured for 96 hr in media containing 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} *M* forskolin or vehicle alone. Media were changed at 48 hr. Values shown represent the mean media calcium concentrations for 5 half calvaria per group at 96 hr incubation. Vertical bars are SE. NS: not significantly different from control group ($P > 0.05$); ***significant at $P < 0.001$, when compared to control group. (□) Control group; (▨) forskolin group.

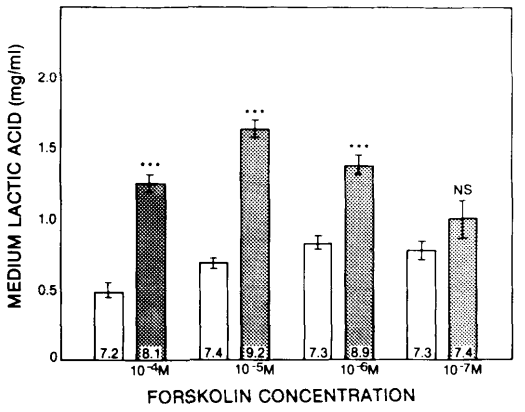

FIG. 2. Lactate release by cultured neonatal mouse half calvaria in response to forskolin. Half calvaria were cultured for 96 hr in media containing 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} *M* forskolin or vehicle alone. Media were changed at 48 hr. Lactic acid and calcium concentrations were determined in the media after 96 hr of incubation. Values shown are mean media lactic acid concentrations for 5 half calvaria per group at 96 hr. Vertical bars are SE. The values inside the bars represent the mean media calcium concentrations during the same period of incubation. NS: not significantly different from control group ($P > 0.05$); ***significant at $P < 0.001$, when compared to control group. (□) Control group; (□) forskolin group.

Carbonic anhydrase activity was assayed using the changing pH method of Wilbur and Anderson (9). The assay buffer was pH 8.2, 0.0167 *M* sodium phosphate buffer containing 0.003% EDTA (10). An Orion Model 801A digital ionalyzer fitted with a Sigma combination pH electrode was used to determine the endpoint of pH 6.5 (11). Activity units (Wilbur–Anderson units) were calculated according to Rickli *et al.* (12).

Statistical analyses. Statistical comparisons between groups were made using Student's *t* test, or, where appropriate, analysis of variance followed by Duncan's multiple range test.

Results. *Bone resorption.* After 96 hr of incubation, forskolin, at concentrations between 1×10^{-4} and 1×10^{-6} *M*, increased calcium release into the medium, indicating enhanced bone resorption. The maximal effect was seen at 1×10^{-5} *M*, and no effect was detected at 1×10^{-7} *M* (Fig. 1).

Lactic acid release. During the first 48 hr of incubation, only cultures containing 1×10^{-6} *M* forskolin exhibited a significant increase in lactic acid release; the latter was accompanied by calcium release into the medium. During the 96-hr incubation period, the

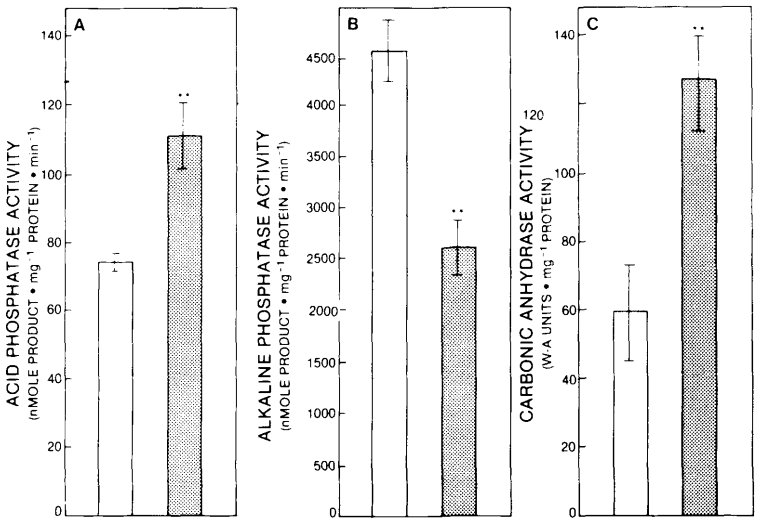

FIG. 3. Skeletal enzyme contents of half calvaria after 96 hr incubation. Half calvaria were treated either with 1×10^{-5} *M* forskolin or vehicle for 96 hr. The media were changed at 48 hr. Twenty-five half calvaria within each group (control or forskolin treated) were pooled prior to homogenization to yield five groups with 5 bones per group. The means of the values obtained from these groups are presented together with SE (vertical bars). Acid phosphatase is shown in (A), alkaline phosphatase in (B), and carbonic anhydrase in (C). **significant at $P < 0.01$ when compared to control group.

effects of forskolin on lactic acid concentration paralleled the calcium release from the bone. At a concentration of 1×10^{-7} *M*, forskolin again caused no enhancement of bone resorption and no increase in lactic acid release (Fig. 2).

Bone enzymes. In a separate set of experiments the enzyme activities were determined in the bone homogenates after 96 hr of treatment. The acid phosphatase activity in the 1×10^{-5} *M* forskolin-treated bones was significantly higher than in the control bones, whereas the alkaline phosphatase activity in the 1×10^{-5} *M* forskolin-treated bones was lower than that of controls. The carbonic anhydrase activity of the bones treated with 1×10^{-5} *M* forskolin was significantly increased more than twofold (Fig. 3).

Acetazolamide inhibition. In the final set of experiments the effect of the carbonic anhydrase inhibitor acetazolamide on the forskolin-induced bone resorption was examined. Forskolin was used at 10^{-5} *M;* acetazolamide was added at 10^{-4} *M*. Forskolin exhibited the expected marked rise in medium calcium at 96 hr (Fig. 4). Acetazolamide significantly, although not completely, blocked this calcium-mobilizing effect of forskolin (Fig. 4), suggesting a role for carbonic anhydrase in this action.

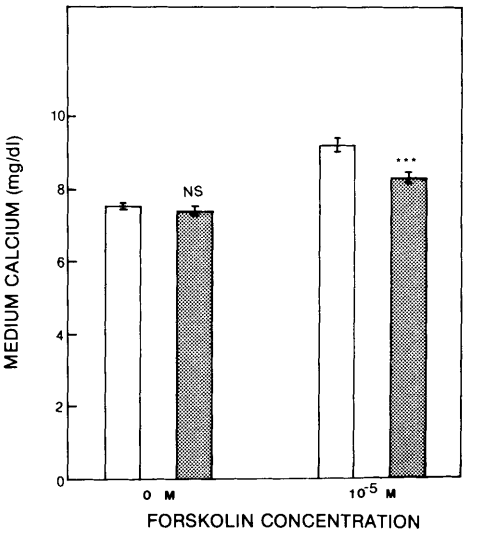

FIG. 4. Effect of acetazolamide on forskolin-induced calcium release. Half calvaria were cultured for 96 hr in media containing vehicle or 1×10^{-5} *M* (□) forskolin or forskolin at this concentration in the presence of 1×10^{-4} *M* (□) acetazolamide. The media were changed at 48 hr. The values shown are mean media calcium concentrations of 5 half calvaria per group at 96 hr incubation. The vertical bars are SE. NS: not significantly different from control group (no forskolin, no acetazolamide; $P > 0.05$); ***significant at $P < 0.001$, when compared to acetazolamide control (no forskolin) group. Forskolin significantly ($P < 0.01$) increased the medium calcium in the acetazolamide-treated cultures indicating that inhibition was incomplete.

Discussion. We have demonstrated that forskolin, at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} *M*, increases the medium calcium, indicating net bone resorption, in neonatal mouse calvaria maintained in culture for 96 hr (Fig. 1).

The release of citrate and lactate into the medium in association with PTH-induced bone resorption has been well documented (13). The present study demonstrated a similar relationship between lactic acid release and forskolin-induced bone resorption in the neonatal mouse calvarium *in vitro* model. Bone resorption was always associated with lactic acid release at 96 hr of incubation; the amount of lactic acid released paralleled the amount of calcium released into the medium (Fig. 2). Forskolin at 1×10^{-7} *M* caused neither bone resorption nor increased lactic acid production.

We have reported elsewhere that PTH- or 1,25-dihydroxyvitamin D_3-induced bone resorption in mouse calvaria *in vitro* is accompanied by an increase in bone carbonic anhydrase activity (14, 15). We and others have also presented evidence to suggest that bone resorption induced by various calcemic agents involves the mediation of carbonic anhydrase (15–22). Important evidence includes the localization of carbonic anhydrase within the bone-resorbing osteoclasts (22) and the ability of the carbonic anhydrase inhibitor acetazolamide to block either bone resorption *in vitro* or hypercalcemia *in vivo* induced by calcemic agents such as PTH, 1,25-dihydroxyvitamin D_3, DBcAMP, and prostaglandin E_2 (15–21). The present study adds further support to the concept that carbonic anhydrase plays a significant role in the mediation of bone resorption. This support is based upon the observations that forskolin, a stimulator of cAMP production, increased bone carbonic anhydrase activity (Fig. 3) in association with in-

creased calcium release from bone (Fig. 1) and that acetazolamide blocked the latter (Fig. 4).

We are grateful for the technical assistance of Judith A. Young. This work was supported in part by a Fellowship from the Educational Commission for Foreign Medical Graduates (Dr. Gunasekaran), an NIH National Research Service Award AM-07124 (Dr. Hall), and an NIH Research Grant AM-19475 (Dr. Kenny). Dr. Gunasekaran performed this work while on a leave of absence from the Department of Physiology, Christian Medical College, Vellore, India.

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Received May 7, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted November 20, 1985.