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Use of forskolin to study the relationship between cyclic AMP formation and bone resorption *in vitro*

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The effect of the adenylate cyclase activator forskolin on bone resorption and cyclic AMP accumulation was studied in an organ-culture system by using calvarial bones from 6–7-day-old mice. Forskolin caused a rapid and fully reversible increase of cyclic AMP, which was maximal after 20–30 min. The phosphodiesterase inhibitor rolipram (30 μmol/l), enhanced the cyclic AMP response to forskolin (50 μmol/l) from a net cyclic AMP response of 1234 ± 154 pmol/bone to 2854 ± 193 pmol/bone (mean $\pm$ S.E.M., $n = 4$). The cyclic AMP level in bones treated with forskolin (30 μmol/l) was significantly increased after 24 h of culture. Forskolin, at and above 0.3 μmol/l, in the absence and the presence of rolipram (30 μmol/l), caused a dose-dependent cyclic AMP accumulation with an calculated EC_{50} (concentration producing half-maximal stimulation) value at 8.3 μmol/l. In 24 h cultures forskolin inhibited spontaneous and PTH (parathyroid hormone)-stimulated ^{45}Ca release with calculated IC_{50} (concentration producing half-maximal inhibition) values at 1.6 and 0.6 μmol/l respectively. Forskolin significantly inhibited the release of ^{3}H from [^{3}H]proline-labelled bones stimulated by PTH (10 nmol/l). The inhibitory effect by forskolin on PTH-stimulated ^{45}Ca release was significant already after 3 h of culture. In 24 h cultures forskolin (3 μmol/l) significantly inhibited ^{45}Ca release also from bones stimulated by prostaglandin E_2 (1 μmol/l) and 1α-hydroxycholecalciferol (0.1 μmol/l). The inhibitory effect of forskolin on spontaneous and PTH-stimulated ^{45}Ca release was transient. A dose-dependent stimulation of basal ^{45}Ca release was seen in 120 h cultures, at and above 3 nmol of forskolin/l, with a calculated EC_{50} value at 16 nmol/l. The stimulatory effect of forskolin (1 μmol/l) could be inhibited by calcitonin (0.1 unit/ml), but was insensitive to indomethacin (1 μmol/l). Forskolin increased the release of ^{3}H from [^{3}H]proline-labelled bones cultured for 120 h and decreased the amount of hydroxyproline in bones after culture. Forskolin inhibited PTH-stimulated release of Ca^{2+}, P_i, β-glucuronidase and β-*N*-acetylglucosaminidase in 24 h cultures. In 120 h cultures forskolin stimulated the basal release of minerals and lysosomal enzymes. In osteoblast-like cells, isolated by enzyme digestion from 2–3-day-old mice, forskolin caused a rapid cyclic AMP response, which was maximal at 15 min. These data show that an increase of cyclic AMP levels in bone by forskolin causes a transient inhibition of bone resorption and support the view that cyclic AMP may be a mediator of the action of calcitonin. The results indicate that the rapid stimulation by PTH on bone resorption is not mediated by cyclic AMP. Finally the data suggest that the PTH-induced increase of cyclic AMP may be involved in a delayed action of the hormone, presumably related to recruitment of new osteoclasts.

INTRODUCTION

Cyclic AMP has been implicated as a second messenger for the effects of parathyroid hormone (PTH), prostaglandin E_2 (PGE_2) and calcitonin (CT) on bone resorption (for a review, see Peck & Klahr, 1979). However, this nucleotide only partially mimics the bone-resorptive effect of PTH and PGE_2 inasmuch as a stimulation of bone resorption, in cultured mouse calvarial bones as well as in fetal-rat long bones, by dibutyryl cyclic AMP and phosphodiesterase (PDE) inhibitors can only be seen after a delay of 24–48 h (Lerner & Gustafson, 1981; McLeod & Raisz, 1981; Lerner et al., 1986*a*). In short-term cultures ($\leq$ 24 h), cyclic AMP analogues and methylxanthines cause a transient inhibition of mineral mobilization (Herrman-Erlee & van der Meer, 1974; Lerner, 1980*a*). These results suggest that the inhibitory action of CT may be mediated intracellularly by cyclic AMP, but that the initial stimulatory effect by PTH and PGE_2 is not.

Forskolin is a naturally occurring diterpene of the labdane family, obtained from the roots of the plant *Coleus forskolii*. It is a potent and reversible activator of most hormonally responsive adenylate cyclases and stimulates cyclic AMP accumulation in broken-cell preparations and intact tissues (Seamon & Daly 1981*a*). The activation of adenylate cyclase by forskolin can be achieved without a functional guanine-nucleotide-binding subunit (Seamon & Daly, 1981*b*; Neer, 1978), but recent data suggest that the subunit is required for

Abbreviations used: PTH, parathyroid hormone; PGE_2 and PGI_2 prostaglandins E_2 and I_2; CT, calcitonin; 1α(OH)D_3, 1α-hydroxycholecalciferol; LDH, lactate dehydrogenase; IBMX, 3-isobutylmethylxanthine; FCS, fetal-calf serum; PBS; phosphate-buffered saline, pH 7.5 (mmol/litre: $CaCl_2$, 0.9; KCl, 3.7; KH_2PO_4, 1.7; $MgCl_2$, 1.1; NaCl, 137; Na_2HPO_4, 8.1); PDE, phosphodiesterase.

a maximal response and for effects at low forskolin concentrations (Darfler *et al.*, 1982; Seamon & Daly, 1985).

We have recently found that forskolin increases cyclic AMP levels in cultured neonatal mouse calvaria and produces a transient inhibition of bone resorption followed by a delayed stimulation (Lerner *et al.*, 1984). In a preliminary report Lorenzo & Nishimoto (1982) found that forskolin, at high concentrations, inhibited bone resorption in fetal-rat long bones, both in short-term and long-term experiments. At lower concentrations forskolin stimulated bone resorption in long-term experiments. Martz & Thomas (1983) have found that forskolin can stimulate cyclic AMP accumulation in chick-embryo tibiae *in vitro* and that forskolin causes a biphasic stimulatory effect on Ca^{2+} efflux from these bones. Recently, Löwik *et al.* (1985) reported that forskolin is a weak transient stimulator of Ca^{2+} release from fetal-mouse calvaria, although it induced a cyclic AMP response comparable with that induced by PTH. In order to investigate further the role of cyclic AMP in the hormonal response in bone tissue we have performed the present study with forskolin.

EXPERIMENTAL

Materials

Forskolin (from *Coleus forskolii*, 7β-acetoxy-8,13-epoxy-1,6β,9-trihydroxy-labd-14-ene-11-one) was purchased from Calbiochem–Behring Corp. Diagnostics, La Jolla, CA, U.S.A. CMRL 1066 and α-MEM (Minimal Essential Medium) media and fetal-calf serum were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. $^{45}CaCl_2$ (sp. radioactivity 20–40 Ci/g), [^{3}H]adenosine 3′,5′-cyclic monophosphate (26–38.5 Ci/mmol), Aquasol-2 and the radioimmunoassay kit for cyclic AMP were obtained from New England Nuclear Chemicals G.m.b.H., Dreieich, Germany. L-[5-^{3}H]Proline (37 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Essentially fatty-acid-free bovine serum albumin (fraction V), phenolphthalein glucuronidate, *p*-nitrophenyl *N*-acetyl-D-glucosaminide and PGE_2 were purchased from Sigma. Synthetic bovine parathyroid hormone (PTH 1–34) with a potency of 6800 i.u./mg was from Beckman, Geneva, Switzerland. Indomethacin was kindly supplied by Merck, Sharp and Dohme, Haarlem, The Netherlands. Synthetic salmon calcitonin with a potency of 5000/mg was kindly given by Sandoz AB, Basel, Switzerland. 1α(OH)D_3 was generously given by Leo Pharmaceutical Products, Copenhagen, Denmark. Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone; ZK 62.711] was kindly donated by Dr. Sprzgala of Schering AG, West Berlin, Germany. 3-isobutylmethylxanthine (IBMX) was from Aldrich Chemicals Co., Milwaukee, WI, U.S.A. Collagenase type 1 (179 units/mg) was obtained from Worthington. All other chemicals were of reagent-grade quality. PGE_2, 1α(OH)D_3, forskolin and indomethacin were dissolved in ethanol and then diluted in medium to stated concentrations. The final concentration of ethanol did not exceed 0.1% and this amount had no effect on basal ^{45}Ca release nor on cyclic AMP levels in calvarial bones or isolated osteoblasts. PTH was dissolved, at a concentration of 10 μmol/l, in 1 mM-HCl containing 1 mg of albumin/ml and stored at −80 °C. The vehicle did not influence basal release of ^{45}Ca. CT was dissolved as a stock solution (10 units/ml) in medium and stored at −80 °C. In most experiments forskolin was dissolved in ethanol just before incubation and diluted in culture medium to the appropriate concentration. However, in some experiments a stock solution of forskolin (10–30 mmol/l), stored at −20 °C, was used.

Methods

Measurements of bone resorption. The organ-culture technique used was that originally described by Reynolds (1976). Calvarial bones (os frontale and parietale) were dissected from 6–7-day-old CsA mice, prelabelled with 1.5 μCi of ^{45}Ca 4 days before the mice were killed (in the kinetic studies the mice were injected with 5 or 12.5 μCi of ^{45}Ca). The calvaria were washed in ice-cold Tyrode's solution and divided along the sagittal suture into two halves. In most experiments calvaria from four to six different litters were pooled and randomized into different groups according to the experimental protocol. The half calvaria were cultured separately on stainless-steel grids in plastic dishes (A/S Nunc, Copenhagen, Denmark) containing 5.5 ml of CMRL 1066 medium modified as described by Lerner & Gustafson (1979). The cultures were gassed with CO_2/air (1:19) at 37 °C. In some experiments (see the legends to the figures) bones were precultured in indomethacin (1 μmol/l) for 24 h (10 calvarial halves/10 ml of medium). After preculture the bones were washed three times in Tyrode's solution and further washed for 3 h in basic medium. Subsequently the bones were transferred to grids and cultured for the stated time periods with or without test substances. The preincubation technique improves the response to PTH and PGE_2 by 75–100% as compared with the response in bones not precultured (U. H. Lerner, unpublished work). After culture the bones were dissolved in 6 M-HCl and samples of the culture media and bones were analysed for radioactivity. The ^{45}Ca release to culture medium was calculated as a percentage of initial radioactivity in bones (= bones + media after culture).

In the short-term kinetic studies (Fig. 4 below) the magnitude of mineral mobilization was determined by withdrawal of small amounts of culture medium (50–300 μl) at stated times and ^{45}Ca release calculated as a percentage of initial radioactivity. These experiments were performed with paired half calvarial bones, i.e. one half of the individual calvarium was used as a control bone and the other half as an experimental bone.

The degradation of organic matrix was assessed either by monitoring the release of ^{3}H from calvarial bones prelabelled with [^{3}H]proline or by analysing the amount of hydroxyproline in bones after culture. Newborn CsA mice were injected with 10 μCi of [^{3}H]proline 4 days before the mice were killed and calvarial bones dissected and cultured as described above. After culture the bones were hydrolysed for 20 h at 110 °C in sealed tubes containing 6 M-HCl. The hydrolysate was evaporated, redissolved in water and portions of bone hydrolysates and culture media were analysed for radioactivity in a liquid-scintillation counter. The c.p.m. values were corrected for quenching by using an external standard. Brand & Raisz (1972) found that the release of ^{3}H is closely related to the release of [^{3}H]hydroxyproline, and thus is a good indicator of collagen breakdown. In parallel with these experiments we cultured non-labelled bones in plastic dishes with 5 ml of medium and analysed the amounts of hydroxyproline in hydrolysates of bone

after culture. Hydroxyproline was determined as described by Kivirikko *et al.* (1967).

Measurement of lysosomal- and non-lysosomal-enzyme release. Half calvarial bones from non-labelled 5–7-day-old CsA mice were dissected and cultured as described above, except that the calvaria were cultured in dishes containing 2 ml of medium. The release of lysosomal enzymes from the bones to media was determined by assaying the activity of β-glucuronidase and β-*N*-acetylglucosaminidase in bones (enzymes liberated by treatment with 0.2% Triton X-100 for 24 h at 4 °C) and media after culture. The assays of β-glucuronidase and β-*N*-acetylglucosaminidase were performed by measuring the hydrolysis of the appropriate glycoside of phenolphthalein and *p*-nitrophenyl respectively (Vaes & Jacques, 1965). Lactate dehydrogenase (LDH) was assayed as a marker for cytosolic enzymes. The activity of LDH in bones and media was assayed by monitoring the oxidation rate of NADH at 340 nm and 25 °C (Wroblewski & LaDue, 1955). All the enzyme determinations were carried out under such conditions that the reactions were directly proportional both to amount of enzyme and incubation time.

The magnitude of bone resorption in the non-labelled calvaria was assessed by analysing the concentrations of stable calcium (Ca^{2+}) and P_i in media before and after culture. Ca^{2+} was analysed by atomic-absorption spectrophotometry (Willis, 1970) and P_i by the method of Chen et al. (1956).

Determination of cyclic AMP production in mouse calvaria. Half calvarial bones from non-labelled 5–7-day-old CsA mice were dissected carefully, pooled and randomized into different groups according to the experimental protocol. Bones were preincubated for 30 min in Erlenmeyer flasks containing 2 ml of prewarmed and pregassed CMRL 1066 medium (4 bones/flask), with or without the potent cyclic AMP PDE inhibitor rolipram (Lerner *et al.*, 1986*a*), at a concentration of 30 μmol/l. Subsequently 2 ml of medium, with or without test substances, was added to the flasks and incubation continued for the stated times. Preincubations and incubations were performed in a shaking water bath (40 oscillations/min) at 37 °C. At the end of the incubations bones were quickly transferred to tubes with 0.7 ml of 90% (v/v) propan-1-ol and cyclic AMP extracted for 24 h at room temperature. The calvaria were removed and the extract evaporated (Ng *et al.*, 1979). Cyclic AMP was determined by the competitive binding method of Brown *et al.* (1972).

Determination of cyclic AMP production in isolated osteoblasts. Bone cells were isolated from calvaria from 2–3-day-old mice with the modified sequential enzyme-digestion technique described by Boonekamp *et al.* (1984). On the basis of hormone responsiveness (PTH and CT), populations 6 and 7 show characteristics of osteoblast-like cells. The osteoblast-like cells were suspended in α-MEM with 10% FCS and seeded in Linbro multiwell dishes at a density of 10^4 cells/cm^2 (2×10^4 cells/well) in 0.5 ml of medium and incubated at 37 °C in CO_2/air (1:19). After attachment of cells overnight, fresh medium was added and replaced every 48 h. After 7 days, when the cell layers were confluent, the cells were carefully washed with prewarmed PBS and incubated for 30 min in α-MEM without serum. Subsequently the cells were pretreated with the PDE inhibitor IBMX (0.2 mmol/l) and then challenged with test substances (in α-MEM without serum and with IBMX) at concentrations and times indicated in the legends to the Figures. At the end of the incubations the medium was drawn off and the reactions terminated by adding ice-cold PBS. Cyclic AMP in the cell layer was extracted with 90% (v/v) propan-1-ol for 24 h at 4 °C. The extract was evaporated, redissolved in assay buffer and cyclic AMP analysed by radioimmunoassay using ^{125}I-cyclic AMP as tracer. In one experiment cyclic AMP was also analysed in the culture medium drawn off the cells.

Calculations of EC_{50} and IC_{50} values. The concentrations for half-maximal stimulation (EC_{50}) and half-maximal inhibition (IC_{50}) were obtained by Hill plots. $\log[R/(1-R)]$ was plotted versus log concentration of agonists. R is the response expressed as a value between 1 and 0, where 1 is maximal response. Linear regression was applied to the data and EC_{50} and IC_{50} values were calculated as the intercept with the x-axis.

Statistics. Statistical evaluation of the data was done using Student's t test for unpaired and paired observations.

RESULTS

Effect of forskolin on cyclic AMP levels in mouse calvarial bones

Forskolin, at concentrations of 30 and 50 μmol/l, both in the absence and the presence of PDE inhibitor, caused an abundant and time-dependent stimulation of cyclic AMP formation in mouse calvarial bones (Fig. 1*a*). The stimulatory effect of forskolin was clearly potentiated by the PDE inhibitor rolipram (Fig. 1*a*, inset). After 60 s, forskolin (50 μmol/l), in the presence of rolipram (30 μmol/l), caused a 23-fold stimulation of cyclic AMP levels and after 24 min a maximal response (211-fold increase) was achieved. The stimulatory effect by forskolin on cyclic AMP was rapidly reversible when forksolin was omitted from the incubation medium (results not shown). Although the cyclic AMP levels in calvaria challenged with forskolin (30 μmol/l) declined after 1 h, significantly increased tissue levels of cyclic AMP could still be seen after 24 h of incubation ($P \leqslant 0.01$). The stimulation of cyclic AMP by forskolin, in the absence and the presence of the PDE inhibitor rolipram (30 μmol/l), was dose-dependent (Fig. 2) and as appears from the inset, the threshold for action was close to 0.3 μmol/l. The concentration required for half-maximal stimulation was 8.3 μmol/l, calculated by the use of the Hill plot.

Effects of forskolin on cyclic AMP levels in isolated osteoblast-like cells

Forskolin, at a concentration of 30 μmol/l, in the presence of the PDE inhibitor IBMX, caused a rapid cyclic AMP response in isolated osteoblasts. The cellular levels of cyclic AMP were maximal after 15 min and thereafter declined, reaching basal levels after 4 h (Fig. 1*b*). The response to PTH (10 nmol/l) at the maximal time point (10 min) was of the same magnitude as the maximal response (15 min) to forskolin (30 μmol/l).

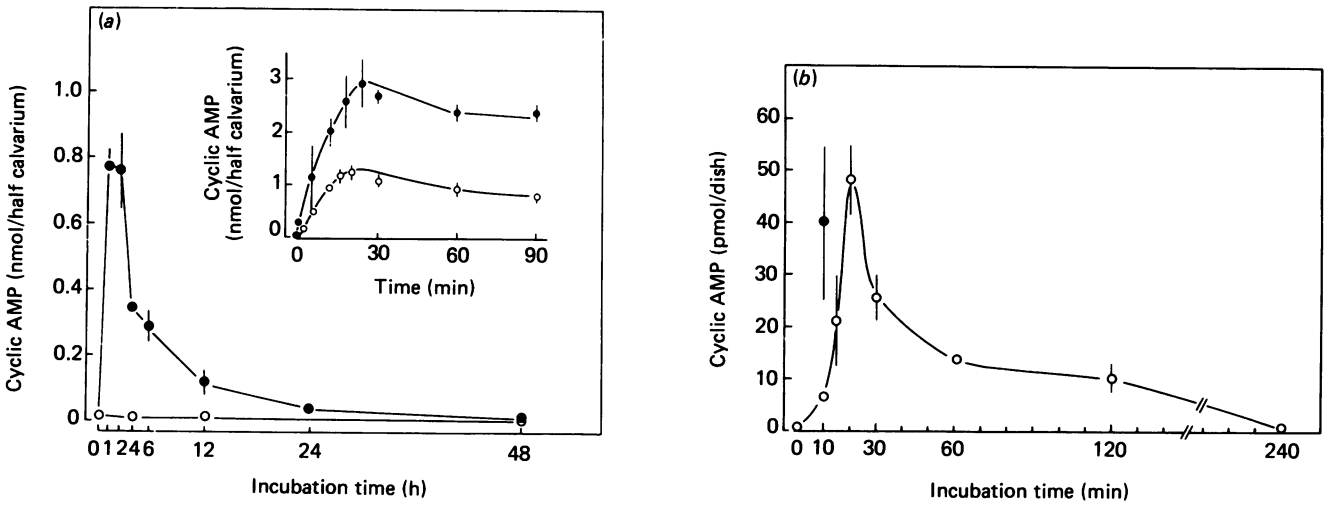

Fig. 1. Time-course study of the effect of forskolin on cyclic AMP accumulation in (*a*) mouse calvarial bones and (*b*) in osteoblast-like cells from neonatal-mouse

(*a*) Bones were preincubated for 30 min in 2 ml of basic medium. Subsequently 2 ml of basic medium (○, controls) or 2 ml of medium with forskolin (final concn. 30 μmol/l, ●) was added and incubation continued for the stated times. The inset shows the effect of forskolin (50 μmol/l), in the absence (○) or the presence (●) of rolipram (30 μmol/l), on cyclic AMP accumulation in mouse calvarial bones. Bones were preincubated for 30 min in 2 ml of medium with and without rolipram (30 μmol/l). Subsequently 2 ml of medium with forskolin (final concn. 50 μmol/l), in the absence and the presence of rolipram (30 μmol/l), was added. Points represent means for four unpaired calvarial bones. The S.E.M. is shown as a vertical bar when this is greater than the radius of the symbol. (*b*) Osteoblast-like cells were isolated from mouse calvaria and cultured in 2 cm^2 plastic dishes until the cells were 80–90% confluent. After a wash and preincubation in IBMX (0.2 mmol/l), the cells were challenged with forskolin (30 μmol/l) and IBMX (0.2 mmol/l) (○) or PTH (10 nmol/l) and IBMX (●). At stated time points cyclic AMP in the cell layer was assayed. Points represent means for four dishes, and the S.E.M. is shown as a vertical bar when it is greater than the radius of the symbol.

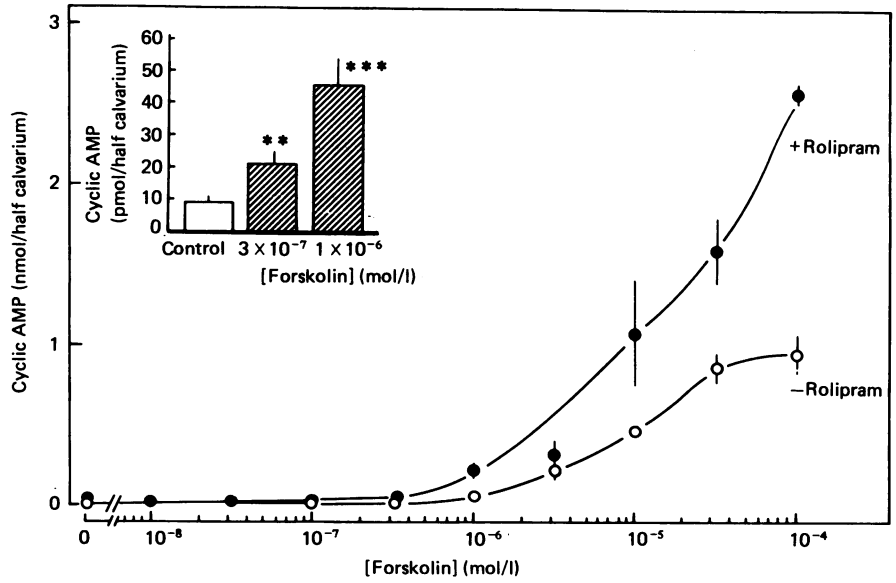

Fig. 2. Effect of forskolin at different concentrations on cyclic AMP levels in mouse calvarial bones

Unlabelled calvarial halves were preincubated in 2 ml of medium for 30 min in the absence (○) or the presence (●) of rolipram (30 μmol/l). After addition of 2 ml of medium with forskolin to the stated final concentrations, in the absence or the presence of rolipram (30 μmol/l), incubation was continued for 30 min. The inset shows the effect of forskolin (0.3 and 1 μmol/l) on cyclic AMP levels in mouse calvarial bones. Points represent means for three unpaired calvarial halves. In the main Figure the S.E.M. is given as vertical bars when it is greater than the radius of the symbol. Significance: **significantly different from control ($P < 0.01$); ***significantly different from control ($P < 0.001$).

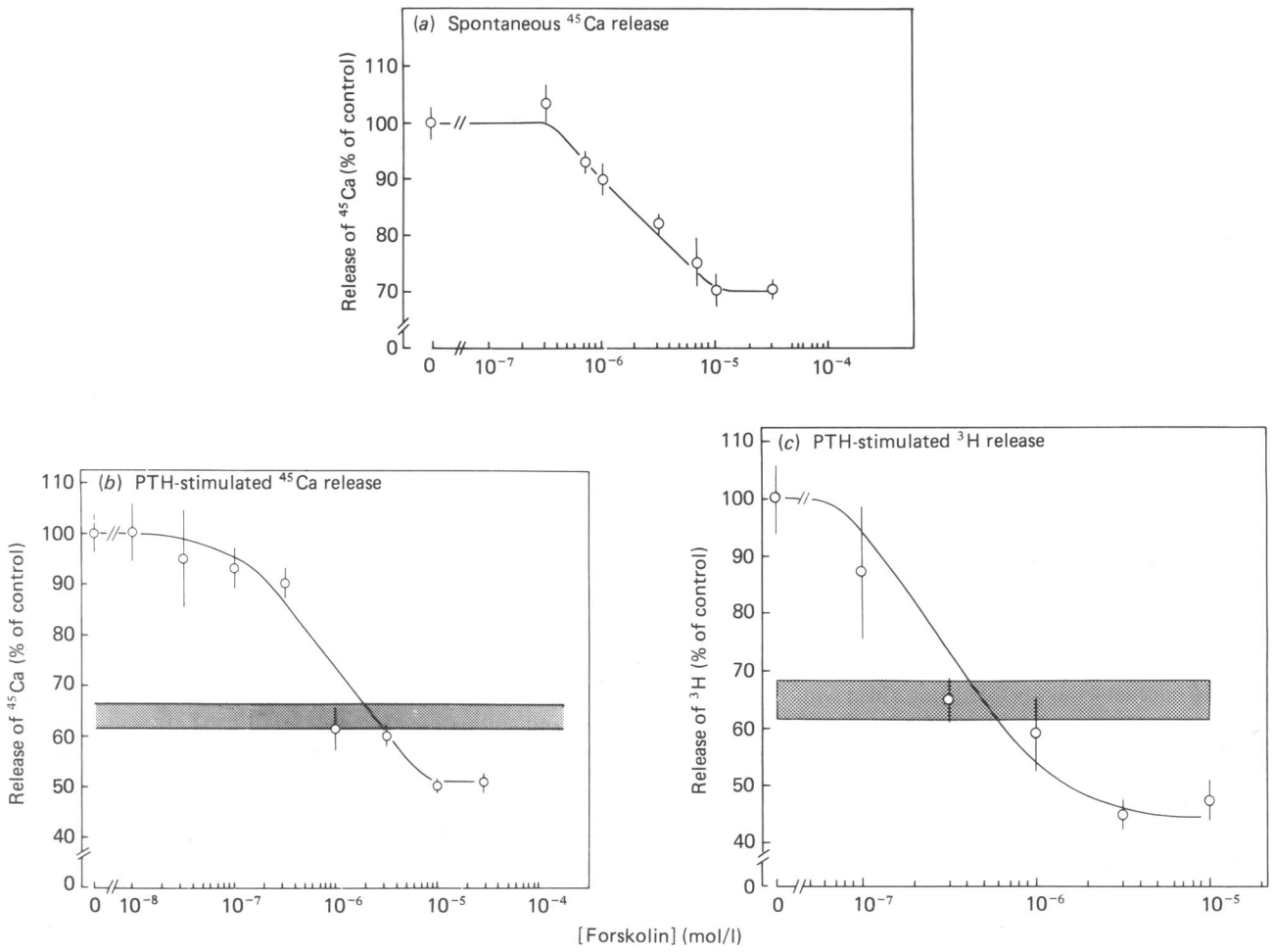

Fig. 3. Effect of different concentrations of forskolin on spontaneous release of ^{45}Ca and on PTH-stimulated release of ^{45}Ca and ^{3}H from mouse calvarial bones cultured for 24 h

Prelabelled calvarial bones (1.5 μCi of ^{45}Ca or 10 μCi of [^{3}H]proline/mouse) were dissected and cultured in the presence of different concentrations of forskolin. After 24 h of culture the radioactivity in the bones and media was analysed and percentage release of initial radioactivity was calculated. Values are given as percentages of ^{45}Ca release from untreated control bones (*a*) or the percentage of ^{45}Ca and ^{3}H release from PTH-stimulated bones (*b*,*c*) (arbitrarily set to 100%). The curve in (*a*) is based on two independent experiments, and the untreated control bones released 22.7±1.0 and 25.0±1.0% of initial ^{45}Ca (mean±S.E.M., $n = 5$). In (*b*), data from three independent experiments are presented. The bones treated only with PTH released 23.8±0.9, 17.0±2.1 and 23.9±0.3% (mean±S.E.M., $n = 5$) of initial ^{45}Ca, respectively. In (*c*) the control bones (treated with PTH; 10^{-8} mol/l) released 14.2±0.5% (mean±S.E.M., $n = 4$) of initial ^{3}H. Points represent means of 5–15 unpaired bones (Figs. 3*a* and 3*b*) or four to five unpaired calvarial halves (*c*). The S.E.M. is shown as a vertical bar. The hatched area indicates ^{45}Ca (*b*) and ^{3}H (*c*) release (mean±S.E.M.) from bones cultured in basic medium.

Effects of forskolin on the release of ^{45}Ca and ^{3}H from mouse calvarial bones cultured for 24 h

Forskolin caused a dose-dependent inhibition of spontaneous ^{45}Ca release from bones cultured for 24 h (Fig. 3*a*) and the calculated IC_{50} value was 1.6 μmol of forskolin/l. Maximal inhibition was seen at 10 μmol/l. Fig. 3(*b*) demonstrates that the inhibitory effect by forskolin on ^{45}Ca release in 24 h cultures could also be seen when bone resorption was stimulated by PTH (10 nmol/l). The calculated IC_{50} value in these experiments was 0.6 μmol/l, and at concentrations of 10 μmol/l the effect of forskolin was maximal. PTH-stimulated release of ^{3}H from [^{3}H]proline-labelled bones was significantly and dose-dependently inhibited by forskolin at and above 0.3 μmol/l (Fig. 3*c*). An inhibitory effect on PTH-stimulated ^{45}Ca release by forskolin (10 and 1 μmol/l) could already be seen after 3 h of incubation (Fig. 4*a*). An inhibition of forskolin (10 μmol/l) could also be seen in calvarial bones prestimulated by PTH (10 nmol/l) for 24 h (Fig. 4*b*). The inhibition by forskolin (10 μmol/l) in pre-stimulated bones was of the same magnitude as the inhibition by CT (100 munit/ml).

The inhibition of ^{45}Ca mobilization by forskolin could be seen not only in PTH-stimulated bones but also in bones stimulated by 1α(OH)D_3 and PGE_2 (Fig. 5). Incubation with PTH (10 nmol/l), 1α(OH)D_3 (0.1 μmol/l) and PGE_2 (1 μmol/l) stimulated ^{45}Ca release from bones 1.6-, 1.4- and 1.6-fold respectively as compared with control bones. The addition of forskolin

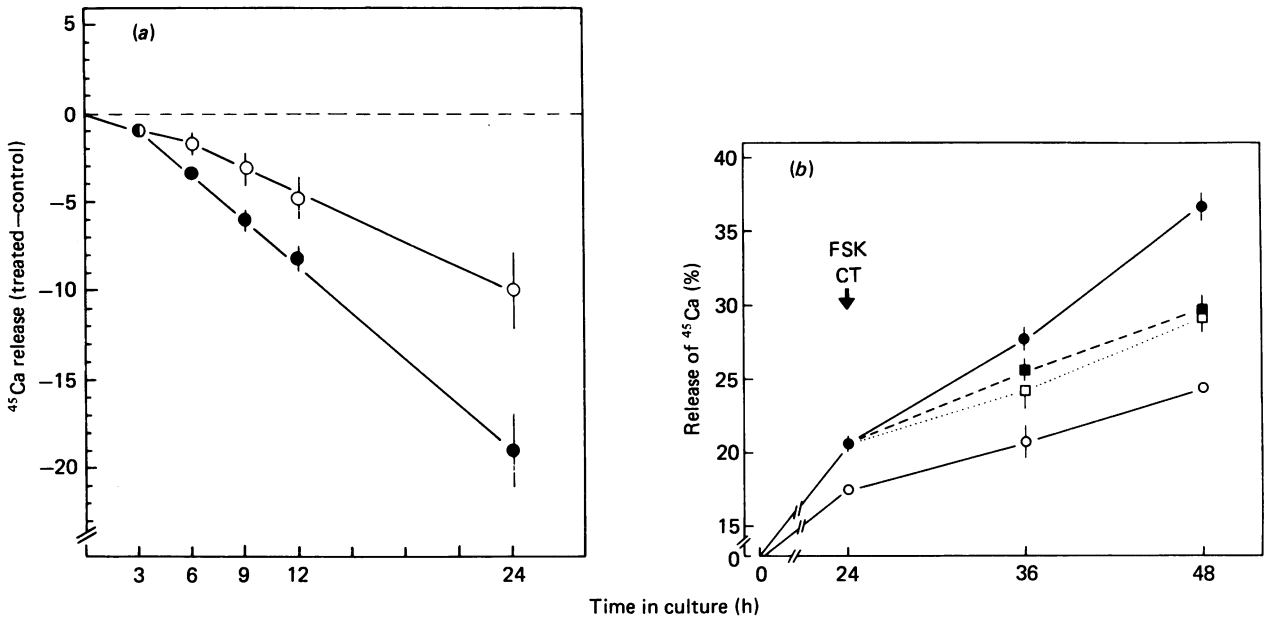

Fig. 4. Time-course study of the effect of forskolin on PTH-stimulated release of ^{45}Ca from mouse calvarial bones

Mice were injected 4 days before being killed with 12.5 μCi of ^{45}Ca. Calvarial bones were dissected and divided into two halves. In the experiment shown in (*a*), one half calvaria was used as a control and cultured in the presence of PTH (10 nmol/). The other, experimental, half, was cultured in the presence of PTH (10 nmol/l) and forskolin [10 (●) and 1 (○) μmol/l]. In the experiment shown in (*b*), calvarial bones were pooled and preincubated in indomethacin as described under 'Methods'. The bones were randomized into four groups of which one was cultured in basic medium and the others were challenged with PTH (10 nmol/l). After 24 h of culture, forskolin (final concn. 10 μmol/l) or calcitonin (final concn. 0.1 unit/ml) were added to two groups of PTH-stimulated bones. In both experiments the release of radioisotope at stated time intervals was determined after withdrawal of small amounts of medium, and after 24 (*a*) or 48 (*b*) h of culture the amount of ^{45}Ca in bones was determined and the percentage release of initial radioactivity at all time points was calculated. In (*a*) values are given as the difference of ^{45}Ca release between forskolin-treated bones and untreated calvarial halves (treated−control). Values represent means for five paired calvarial halves and the S.E.M. is given as a vertical bar when it is greater than the radius of the symbol. After 3 h of culture the release of ^{45}Ca from treated bones was significantly different from control bones ($P < 0.05$; *a*). In (*b*), values represent the means for six to seven unpaired calvarial halves. The S.E.M. is given as a vertical bar when it is greater than the radius of the symbol. After 24 h of treatment the release of ^{45}Ca from PTH-stimulated bones treated with forskolin (FSK) (■) or CT (□) was significantly different from bones treated with PTH alone (●) ($P \leqslant 0.01$). ○, Control.

(3 μmol/l) caused a 30% decrease in ^{45}Ca release in PTH-stimulated bones and a 33% and 41% decrease in $1\alpha(OH)D_3$- and PGE_2-stimulated bones respectively.

Effects of forskolin on the release of ^{45}Ca and the degradation of matrix in mouse calvarial bones cultured for 120 h

The kinetic study presented in Fig. 6 demonstrates that the inhibition of ^{45}Ca release by forskolin in short-term cultures was transient and that a stimulation of ^{45}Ca release could be seen when the incubation time was extended to 120 h. The inhibition was maximal after 24 h, irrespective of the degree of inhibition. Forskolin-treated bones started to release ^{45}Ca at this time point.

The stimulatory effect of forskolin on ^{45}Ca release in 120 h cultures was dose-dependent, with the threshold for action being 3 nmol/l ($P \leqslant 0.05$; Fig. 7). The calculated EC_{50} value was 16 nmol/l and maximal stimulation was obtained at 1 μmol/l (1.5-fold stimulation). The dose–response curve was biphasic, and above 3 μmol of forskolin/l the stimulatory effect declined. Forskolin had no effect on ^{45}Ca release from bones devitalized by heating at 70 °C for 5 min in culture medium (results not shown).

Forskolin significantly and dose-dependently increased the release of ^{3}H from [^{3}H]proline-labelled calvaria cultured for 120 h (Fig. 8). The degree of stimulation was comparable with that obtained on mineral mobilization. In agreement with the curve for ^{45}Ca release, the effect on ^{3}H release declined at 10 μmol/l. At the end of a 120 h culture period, the amount of hydroxyproline in control bones was 39.9 ± 2.0 μg/half calvaria (mean ± S.E.M.; $n = 7$). In bones treated with PTH (10 nmol/l) or forskolin (1 μmol/l) for 120 h the amount of hydroxyproline was significantly ($P \leqslant 0.001$) decreased to 14.1 ± 2.3 and 23.1 ± 2.1 μg/half calvaria (mean ± S.E.M.; $n = 7$–8) respectively.

The stimulatory effect of forskolin (3 μmol/l) on ^{45}Ca release in 96 h cultures was significantly inhibited by addition of CT (0.1 unit/ml). By contrast, CT did not significantly inhibit ^{45}Ca release in control bones (Fig. 9). CT was added to bones prestimulated by forskolin for 48 h, since the inhibitory effect by CT on bone resorption stimulated by PTH declines after 24–48 h ('escape from

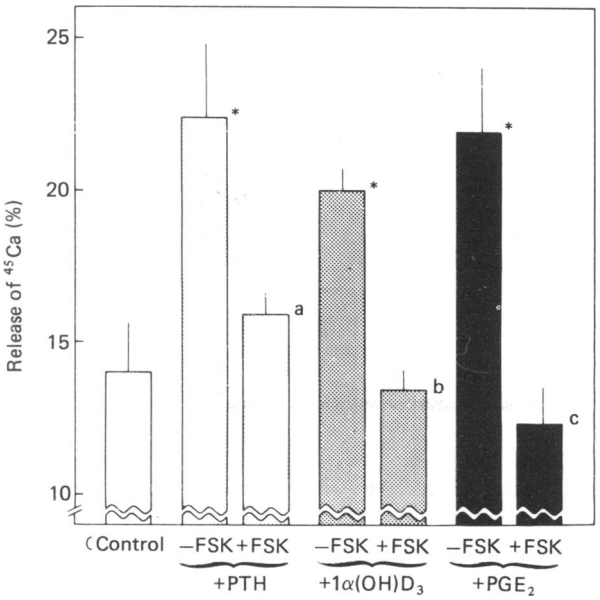

Fig. 5. Effect of forskolin on PTH-, PGE_2- and $1\alpha(OH)D_3$-stimulated ^{45}Ca release from mouse calvarial bones cultured for 24 h

Prelabelled bones (1.5 μCi/mouse) were dissected and calvarial halves were pooled and randomized in six groups and cultured in the presence of PTH (10 nmol/l), PGE_2 (1 μmol/l) or $1\alpha(OH)D_3$ (0.1 μmol/l), with and without forskolin (FSK; 3 μmol/l). After 24 h of culture the bones and media were analysed for their contents of ^{45}Ca and percentage release of initial ^{45}Ca was calculated. Values indicate means for five unpaired calvarial halves and the S.E.M. is given as a vertical bar. Significance: a, significantly different from PTH alone ($P \leqslant 0.05$); b, significantly different from $1\alpha(OH)D_3$ alone ($P \leqslant 0.001$); c, significantly different from PGE_2 alone ($P \leqslant 0.01$); *, significantly different from control ($P \leqslant 0.01$).

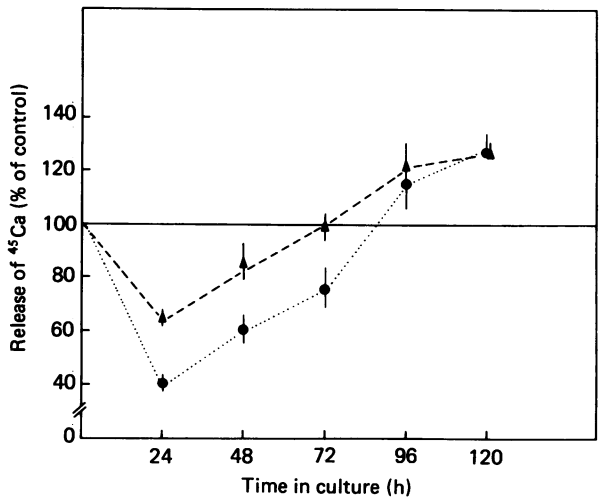

Fig. 6. Time-course study of the effect of different doses of forskolin on ^{45}Ca release from mouse calvarial bones

Radiolabelled bones (5 μCi of ^{45}Ca/mouse) were dissected, preincubated for 24 h in indomethacin and washed as described under 'Methods'. Calvarial bones were pooled and randomized in four groups and cultured for 120 h in basic medium or in the presence of forskolin [3 (●) or 0.3 μmol (▲)/l]. The release of ^{45}Ca at the stated time intervals was determined after withdrawal of small amounts of medium. After 120 h of culture the radioactivity in the bones was analysed and the percentage release of initial ^{45}Ca at all time points was calculated. Points indicate means for five unpaired calvarial halves and the S.E.M. is shown as a vertical bar. The untreated control bones released 17.8 ± 0.5, 31.3 ± 0.8, 39.3 ± 0.6, 40.1 ± 1.1 and $43.3 \pm 0.9\%$ of ^{45}Ca at 24, 48, 72, 96 and 120 h of culture respectively.

inhibition'; Wener *et al.*, 1972). Although we can demonstrate 'escape from inhibition' by CT in PTH-stimulated bones in our system, we have, in three independent experiments, seen inhibition by CT in forskolin-stimulated bones without any sign of 'escape from inhibition'.

Forskolin also caused a dose-dependent stimulation of ^{45}Ca release in 120 h cultures of mouse calvaria treated simultaneously with indomethacin (1 μmol/l; results not shown). In the presence of indomethacin, forskolin (3 μmol/l) caused a 2.19-fold ($P \leqslant 0.001$) stimulation of ^{45}Ca mobilization.

Effect of forskolin on the release of Ca^{2+}, P_i, lysosomal and non-lysosomal enzymes from mouse calvarial bones cultured for 24 and 120 h

PTH (10 nmol/) stimulated the release of Ca^{2+}, P_i, β-glucuronidase and β-*N*-acetylglucosaminidase without affecting the release of LDH in 24 and 120 h cultures (Table 1). In 24 h cultures, forskolin (10 μmol/l) significantly inhibited the release of Ca^{2+}, P_i, β-glucuronidase and β-*N*-acetylglucosaminidase from PTH-stimulated bones (Table 1, expt. 1). In 120 h cultures forskolin (1 μmol/l), in the absence of PTH, significantly stimulated the release of Ca^{2+}, P_i and β-glucuronidase (Table 1, expt. 2). No effect by forskolin on the release of LDH was seen (Table 1, expts. 1 and 2).

DISCUSSION

We have confirmed and extended our previous finding that the adenylate cyclase activator forskolin, both in the absence and the presence of a PDE inhibitor, stimulates cyclic AMP production in cultured mouse calvarial bones (Lerner *et al.*, 1984). The response is dose-dependent, with a threshold for action (cyclic AMP production) at 0.3 μmol of forskolin/l, both in the absence and the presence of the PDE inhibitor rolipram. The EC_{50} value, 8.3 μmol/l, is in good agreement with EC_{50} values reported for forskolin activation of cyclic AMP in most intact cell systems (Seamon & Daly, 1981*a*). The cyclic AMP response in bones incubated with forskolin is as rapid as the response to PTH (within seconds), but the maximal cyclic AMP level is seen after a longer period of time (20–30 min) as compared with the PTH response (5–10 min; U. Lerner, M. Ransjö, K. Sahlberg, O. Ljungren & B. B. Fredholm, unpublished work). Furthermore, the cyclic AMP elevation after forskolin treatment is much more long-lasting and of considerably greater magnitude than the PTH response. The stimulatory effect on cyclic AMP accumulation by forskolin is rapidly reversible. Bones incubated with forskolin, and thereafter washed, show a rapid decline in the cyclic AMP response, but can be restimulated if re-challenged with forskolin. The decline of cyclic AMP levels in bone during continous incubation with forskolin is not an

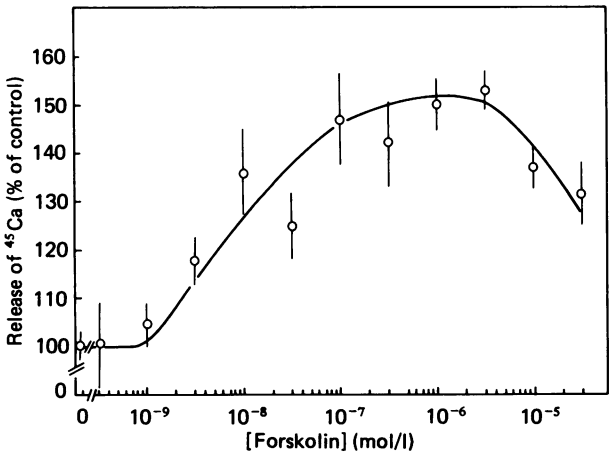

Fig. 7. Effect of different concentrations of forskolin on spontaneous ^{45}Ca release from mouse calvarial bones cultured for 120 h

Radiolabelled calvarial bones (1.5 μCi/mouse) were dissected and calvarial halves cultured with and without forskolin. After 120 h of culture the radioactivity in the bones and media was analysed and release of ^{45}Ca was determined as the percentage of the inital radioactivity. The stimulatory effect is expressed as a percentage of the values obtained with untreated bones, which were set to 100%. Points indicate means for 5–15 unpaired calvarial halves and the S.E.M. is shown as a vertical bar. The percentage ^{45}Ca release from untreated bones in the five independent experiments on which the curve is based was 29.1 ± 2.3, 41.4 ± 1.8, 35.8 ± 1.7, 35.3 ± 2.4 and 36.9 ± 1.2 respectively (mean $\pm$ S.E.M., $n = 5$) of the initial ^{45}Ca. The stimulation of ^{45}Ca release was statistically significant at and above a concentration of 3 nmol of forskolin/l ($P \leqslant 0.05$).

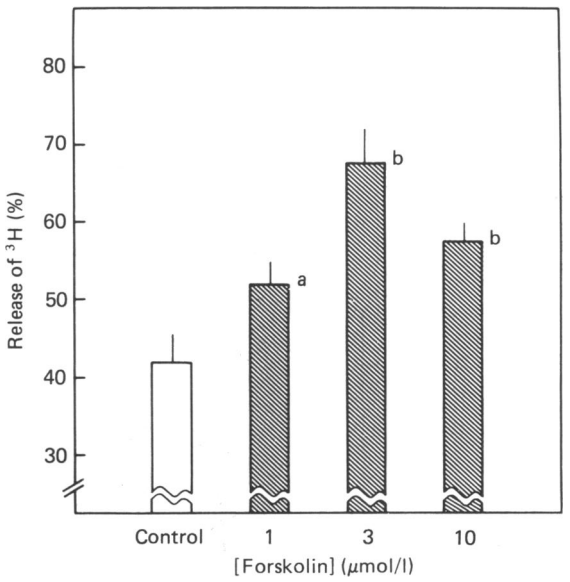

Fig. 8. Effect of different concentrations of forskolin on ^{3}H release from mouse calvarial bones cultured for 120 h

Bones, radiolabelled with [^{3}H]proline (10 μCi/mouse) 4 days before exsanguination, were dissected and calvarial halves were cultured in the absence and the presence of different concentrations of forskolin. After 120 h of culture the amount of radioactivity in the bones and media was analysed and the release of ^{3}H determined as a percentage of initial radioactivity in the bones. Values represent means $\pm$ S.E.M. for five unpaired calvarial halves. a, Significantly different from control ($P \leqslant 0.05$); b, significantly different from control ($P \leqslant 0.01$).

effect of forskolin degradation, since a full cyclic AMP response can be obtained in bones incubated in forskolin-containing medium that has previously been used for a 48 h incubation with mouse calvaria (results not shown).

According to the current view, PTH-stimulated cyclic AMP in bone is due to stimulation of osteoblasts. We therefore analysed the effect by forskolin on cyclic AMP in osteoblasts. Forskolin stimulated cyclic AMP production in osteoblasts isolated from neonatal mouse calvaria. The response was of the same order of magnitude as the response to PTH. As compared with the response in calvaria, forskolin-stimulated cyclic AMP in osteoblasts declined more rapidly. However, when total cyclic AMP (medium and cells) was assayed, the amount of cyclic AMP increased with time for at least 24 h (results not shown). This indicates that forskolin stimulates cyclic AMP production for long periods of time and that the cells acquire the capacity to extrude the nucleotide effectively.

Earlier studies with dibutyryl cyclic AMP (Herrmann-Erlee & van der Meer, 1974; Lerner & Gustafson, 1979; McLeod & Raisz, 1981), PDE inhibitors and cyclic AMP analogues (Lerner, 1980*a*; Herrmann-Erlee & van der Meer, 1974) have demonstrated an inhibitory action of cyclic AMP on both spontaneous and stimulated bone resorption in short-term cultures (0–24 h). We show here that both spontaneous and stimulated bone resorption in 24 h bone cultures is inhibited when cyclic AMP levels are raised in the tissue by forskolin. Our finding that forskolin can inhibit not only PTH- but also PGE_2- and $1\alpha(OH)D_3$-stimulated ^{45}Ca release in 24 h cultures suggests that forskolin exerts its inhibitory action by a mechanism distal to the receptors to these agonists. The inhibitory effect of forskolin on ^{45}Ca release from PTH-stimulated bones was significant after 3 h, indicating that the decreased bone resorption is due to a direct inhibitory effect on the activity of the osteoclasts. In sharp contrast with this inhibition of bone resorption obtained with forskolin is the stimulatory effect by PTH, which in mouse calvaria can be observed after 60 min (Lerner *et al.*, 1985). The effect of forskolin in 24 h cultures was tested over a wide range of concentrations and we never did find any stimulation of radioactive mineral release. The inhibition of ^{45}Ca release was not due to an effect on passive exchange of radioisotope between bone and medium, since forskolin inhibited the mobilization of both Ca^{2+} and P_i from PTH-stimulated calvaria in 24 h cultures. Moreover, forskolin inhibited PTH-stimulated release of ^{3}H from [^{3}H]proline-labelled bones, at the same concentrations as those that inhibited ^{45}Ca release. This observation indicates that not only mineral mobilization, but also organic matrix degradation, is affected by forskolin. Finally, in strong support of our interpretation that forskolin inhibits bone resorption via cyclic AMP, is our recent finding that, in 24 h cultures, forskolin-induced inhibition of PTH-stimulated ^{45}Ca release can be potentiated by several structurally different PDE inhibitors (results not shown).

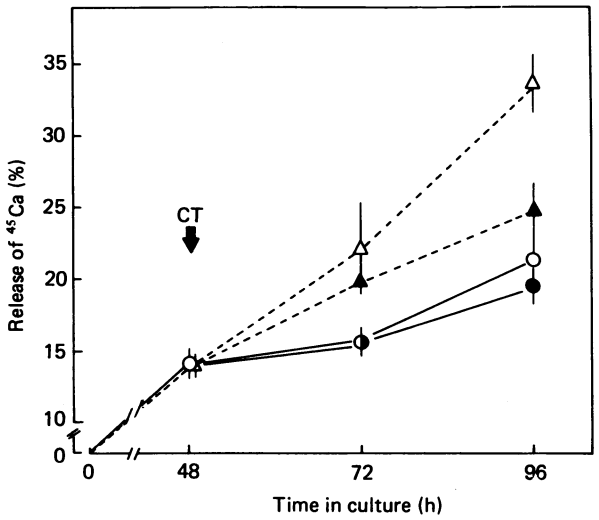

Fig. 9. Effect of CT on forskolin-stimulated ^{45}Ca release from mouse calvarial bones cultured for 96 h

Prelabelled calvarial bones (1.5 μCi of ^{45}Ca/mouse) were dissected, divided into halves, preincubated for 24 h in indomethacin and washed as described under 'Methods'. Subsequently the bones were incubated for 96 h in the absence (control, ○) or in the presence of forskolin (△) (1 μmol/l). At 48 h of culture CT (0.1 unit/ml) was added to one half of the control bones (●) and to one half of the bones stimulated by forskolin (▲). The ^{45}Ca release from bones to media at the stated time intervals was determined after withdrawal of small amounts of media. After 96 h of culture the radioactivity in the bones was analysed and the percentage release of initial ^{45}Ca at all time points was determined. Values represent means ± S.E.M. for five paired calvarial halves.

Previous studies have indicated that there is an intimate relationship between the release of lysosomal enzymes and bone resorption (Eilon & Raisz, 1978; Lerner, 1980*b*,*c*; Vaes, 1980). We show here that forskolin-induced inhibition of bone resorption is associated with decreased lysosomal-enzyme release. Since forskolin has no effect on the release of lactate dehydrogenase, as non-lysosomal enzyme, it seems that the inhibition of lysosomal-enzyme release is caused by an action on a specific degranulation process. This indicates that the effect of forskolin is cell-mediated. Earlier studies have demonstrated that dibutyryl cyclic AMP inhibits lysosomal-enzyme release in parallel with a decreased bone resorption (Lerner & Gustafson, 1979). Moreover, when cyclic AMP, or its analogues, were combined with a PDE inhibitor, the inhibitory effect on lysosomal-enzyme release was potentiated (Lerner, 1980*a*). These results strongly indicate that an increase of cyclic AMP inhibits bone resorption and lysosomal degranulation. A similar inhibitory action of cyclic AMP on lysosomal degranulation has been reported in leukocytes (Zurier *et al.*, 1974).

The inhibitory action of forskolin on bone resorption in short-term cultures (present study) is in agreement with the findings in a preliminary report by Lorenzo & Nishimoto (1982). Those authors reported that, in 2-day fetal-rat long-bone cultures, forskolin (10 μmol/l) inhibited the release of ^{45}Ca. Similarly, Löwik *et al.* (1985) recently reported that forskolin (5 and 10 μmol/l) inhibited Ca^{2+} mobilization from fetal-mouse calvaria. When forskolin was added at a concentration of 0.1 μmol/l, Ca^{2+} mobilization was stimulated in 6 h cultures (Löwik *et al.*, 1985). However, the effect was not sustained, and the magnitude was less than the effect by PTH at a dose giving the same cyclic AMP response. In a study by Martz & Thomas (1983) it was demonstrated that, in 2–8 h incubations, forskolin, at a concentration of 1 μmol/l, stimulated Ca^{2+} efflux from a chick-embryo system. However, in our system the forskolin-induced inhibition of bone resorption is transient (Lerner *et al.*, 1984; the present study), and forskolin produces a dose-dependent stimulation of ^{45}Ca and ^{3}H release in long-term cultures (120 h; the present study). The kinetic studies reveal that, as long as cyclic AMP is elevated in

Table 1. Effect of forskolin on the release of Ca^{2+}, P_i, lysosomal and non-lysosmal enzymes from mouse calvarial bones cultured for 24 and 120 h

Non-labelled calvarial bones were dissected and precultured as described under 'Methods'. Subsequently bones were pooled and randomized into three groups according to experimental protocol. In Expt. 1, bones were cultured for 24 h in the absence or the presence of PTH (10 nmol/l) or in the presence of both PTH and forskolin (10 μmol/l). In Expt. 2, bones were cultured for 120 h, in the absence and the presence of PTH (10 nmol/l) or forskolin (1 μmol/l). After culture the concentrations of Ca^{2+} and P_i in media were determined. The activities of β-glucuronidase, β-*N*-acetylglucosaminidase and lactate dehydrogenase (LDH) were assayed in extracts from bone and culture media and the percentage release (of total) was calculated. Negative values for Ca^{2+} and P_i indicate that there is a net increase of Ca^{2+} and P_i in the bones. This is not an unusual finding in bones with low basal release of Ca^{2+} and P_i. Values are means ± S.E.M. for eight calvarial halves. Statistical significance: [a]significantly different from untreated control ($P \leqslant 0.01$); [b]significantly different from PTH alone ($P \leqslant 0.001$).

Expt.	Additions	Concn. (μmol/l)	Culture time (h)	Ca^{2+} (μg/half calvarium)	P_i (μg/half calvarium)	Activity (% of total) β-Glucuronidase	β-*N*-acetylglucosaminidase	LDH
1	–	–	24	−1.4±1.1	1.4±0.7	4.5±0.6	3.1±0.8	7.8±0.6
	PTH	0.01	24	7.5±1.6[a]	6.5±1.1[a]	10.6±0.9[a]	7.9±1.1[a]	8.8±1.0
	PTH+forskolin	0.01+10	24	−3.4±0.6[b]	−2.4±0.4[b]	5.2±0.5[b]	1.7±0.2[b]	9.0±0.7
2	–	–	120	−2.4±0.8	−3.7±0.8	8.2±0.4	6.5±0.5	10.4±1.1
	PTH	0.01	120	20.4±2.4[a]	6.7±1.4[a]	16.8±1.7[a]	9.4±0.9[a]	13.2±1.5
	Forskolin	1	120	5.3±1.7[a]	1.9±1.0[a]	13.3±1.6[a]	—	12.4±1.4

the bones, there is a decreased rate of bone resorption. When cyclic AMP has declined to basal levels (after 24 h) the bones start to resorb. In the study by Martz & Thomas (1983), the cyclic AMP response was of much shorter duration than in our system. The divergent results on Ca^{2+} mobilization in short-term cultures (< 24 h) is not easily explained. However, the two studies showing an initial stimulatory effect by forskolin (Martz & Thomas, 1983; Löwik *et al.*, 1985) use the release of stable Ca^{2+}, which is the net effect of accretion and resorption processes, as a parameter of bone resorption, whereas we and Lorenzo & Nishimoto (1982) use ^{45}Ca release to quantify the rate of resorption processes.

The dissociation between the dose–response curves for inhibition (IC_{50} 1.6 μmol/l) and stimulation (EC_{50} 16 nmol/l) on mineral mobilization by forskolin indicates that, at low concentrations of forskolin, a delayed stimulation without a preceding inhibition can be obtained, in agreement with findings by Lorenzo & Nishimoto (1982). The stimulatory effect by forskolin in long-term cultures is cell-mediated, as supported by the findings that forskolin not only stimulated mineral mobilization (^{45}Ca, Ca^{2+}, P_i), but also the release of lysosomal enzymes and the degradation of bone matrix (release of ^{3}H from [^{3}H]proline-labelled bones, amounts of hydroxyproline at the end of culture). Furthermore, no stimulation of ^{45}Ca release by forskolin was seen in devitalized bones. Stimulation of ^{45}Ca release was blocked by CT, indicating that the stimulatory effect of forskolin was at least partially osteoclasts-mediated. Indomethacin did not affect the stimulatory effect of forskolin on the release of ^{45}Ca, suggesting that the stimulation is not prostaglandin-mediated. The delayed stimulation of bone resorption and release of lysosomal enzymes produced by the adenylate cyclase stimulator forskolin is in good agreement with the reported effect by dibutytryl cyclic AMP (Herrmann-Erlee & van der Meer, 1974; McLeod & Ralsz, 1981; Lerner & Gustafson, 1981), PDE inhibitors (Herrmann-Erlee & van der Meer, 1974; Lerner & Gustafson, 1981; Lerner *et al.*, 1986*a*) and cholera toxin (M. Ransjö & U. H. Lerner, unpublished work).

An interesting finding in the present study is that the biological effect of forskolin, in long-term cultures, can be seen at concentrations much lower (EC_{50} 16 nmol/l) than those required to induce a measurable cyclic AMP response (EC_{50} 8.3 μmol/l). We have made similar observations using cholera toxin, PTH, PGE_2, CT and PDE inhibitors as stimulants.

The cyclic AMP response to PTH is seen mainly in osteoblast-like cells, whereas the cyclic AMP response to calcitonin is only obtained in osteoclast-like cells (Luben *et al.*, 1976). We suggest that, when cyclic AMP in osteoclasts is elevated by CT, dibutyryl cyclic AMP, cholera toxin and forskolin, there is a decreased bone resorptive activity of the osteoclast. Further support for this view is provided by the work by Chambers & Ali (1983), Chambers & Dunn (1983) and Chambers *et al.* (1985), who demonstrated that CT, PGE_2, PGI_2, dibutyryl cyclic AMP but not PTH, inhibit the motility and bone-resorptive capacity of isolated osteoclasts. In line with the observations in isolated osteoclasts, we have recently found that PGE_2 can acutely inhibit bone resorption in unstimulated mouse calvaria and that this effect can be potentiated by several PDE inhibitors (Lerner *et al.*, 1986*b*). Thus we propose that the initial bone-resorptive effect by PTH is not mediated by cyclic AMP. Instead, as indicated by the studies with forskolin, dibutyryl cyclic AMP, PDE inhibitors and cholera toxin, the nucleotide can be a mediator of the late bone-resorptive effect by PTH. Furthermore, we suggest that the delayed stimulation may be related to an increased number of osteoclasts via an elevation of cyclic AMP in osteoblasts or in osteoclast precursor cells. However, our speculation must await morphological data before it can be accepted.

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REFERENCES

Boonekamp, P. M., Hekkelman, J. W., Hamilton, J. W., Cohn, D. C. & Jilka, R. L. (1984) Proc. K. Ned. Akad. Wet. Ser. B **87**, 371–382

Brand, J. S. & Raisz, L. G. (1972) Endocrinology **90**, 479–487

Brown, B. L., Ekins, R. P. & Albano, J. D. M. (1972) Adv. Cyclic Nucleotide Res. **2**, 25–40

Chambers, T. J. & Ali, N. N. (1983) J. Pathol. **139**, 383–397

Chambers, T. J. & Dunn, T. (1983) Calcif. Tissue Int. **35**, 566–570

Chambers, T. J., McSheehy, P. H. J., Thompson, B. M. & Fuller, K. (1985) Endocrinology (Baltimore) **116**, 234–239

Chen, P. S., Toribara, T. Y. & Warner, H. (1956) Anal. Chem. **28**, 1756–1758

Darfler, F. J., Mahan, L. C., Koachman, A. M. & Insel, P. A. (1982) J. Biol. Chem. **257**, 11901–11907

Eilon, G. & Raisz, L. G. (1978) Endocrinology (Baltimore) **103**, 1969–1975

Hermann-Erlee, M. P. M. & van der Meer, J. M. (1974) Endocrinology (Baltimore) **94**, 424–434

Kivirikko, K. I., Laitinen, O. & Prockop, D. J. (1967) Anal. Biochem. **19**, 249–255

Lerner, U. (1980*a*) Acta Endocrinol. **94**, 138–144

Lerner, U. (1980*b*) Biochem. Biophys. Acta **632**, 204–213

Lerner, U. (1980*c*) Cellular Mechanisms Involved in Bone Resorption (Umeå University Odontological Dissertations; ISSN 0345–7532), University of Umeå, Umeå

Lerner, U. & Gustafson, G. T. (1979) Acta Endocrinol. **91**, 730–742

Lerner, U. & Gustafson, G. T. (1981) Acta Endocrinol. **97**, 281–288

Lerner, U., Fredholm, B. B. & Ransjö, M. (1984) Acta Physiol. Scand. **120**, 159–160

Lerner, U., Fredholm, B. B. & Hänström, L. (1985) J. Oral Pathol. **14**, 644–653

Lerner, U., Fredholm, B. B., Ransjö, M. (1986*a*) Biochem. Pharmacol., in the press

Lerner, U., Ransjö, M. & Ljunggren, Ö. (1986*b*) Calcif. Tissue Int., in the press

Lorenzo, J. & Nishimoto, M. (1982) Calcif. Tissue Int. **34**, suppl. 1, abstr. S10

Löwik, C. W. G. M., van Leeuwen, J. P. T. M., van der Meer, J. M., van Zeeland, J. K., Scheven, B. A. A. & Herrmann-Erlee, M. P. M. (1985) Cell Calcium **6**, 311–326

Luben, R. A., Wong, G. L. & Cohn, D. V. (1976) Endocrinology (Baltimore) **99**, 526–534

Martz, A. & Thomas, M. L. (1983) Biochem. Pharmacol. **32**, 3429–3433

McLeod, J. F. & Raisz, L. G. (1981) Endocr. Res. Commun. **8**, 49–59

Neer, E. J. (1978) J. Biol. Chem. **253**, 5808–5812

Ng, B., Hekkelmann, J. W. & Heersche, J. N. M. (1979) Endocrinology (Baltimore) **104**, 1130–1135
Peck, W. A. & Klahr, S. (1979) Adv. Cyclic Nucleotide Res. **10**, 89–130
Reynolds, J. J. (1976) In Organ Culture in Biomedical Research (Balls, M. & Monnickendam, M., eds.), pp. 355–366. Cambridge University Press, Cambridge
Seamon, K. B. & Daly, J. W. (1981*a*) J. Cyclic Nucleotide Res. **7**, 201–224
Seamon, K. B. & Daly, J. W. (1981*b*) J. Biol. Chem. **256**, 9799–9801
Seamon, K. B. & Daly, J. W. (1985) Adv. Cyclic Nucleotide and Protein Phosphorylation Res. **19**, 125–137
Vaes, G. (1980) in Collagenases in Normal and Pathological Connective Tissues (Woolley, D. E. & Evanson, J. M., eds.), pp. 185–207, John Wiley and Sons, Chichester
Vaes, G. & Jacques, P. (1965) Biochem. J. **97**, 380–388
Wener, J. A., Gorton, S. J. & Raisz, L. G. (1972) Endocrinology (Baltimore) **90**, 752–759
Willis, J. B. (1970) in Analytical Flame Spectroscopy (Mavrodineanu, R., ed.), pp. 525–594, Philips Technical Library, Eindhoven
Wroblewski, F. & LaDue, J. S. (1955) Proc. Soc. Exp. Biol. Med. **90**, 210–213
Zurier, R. B., Weissman, G., Hoffstein, S., Kammerman, S. & Tai, H. H. (1974) J. Clin. Invest. **53**, 297–309

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