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COMPARATIVE STUDY OF THE EFFECTS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE INHIBITORS ON BONE RESORPTION AND CYCLIC AMP FORMATION *IN VITRO*

ULF LERNER,*† MARIA RANSJÖ* and BERTIL B. FREDHOLM‡

*Department of Oral Pathology, University of Umeå, S-901 87 Umeå, Sweden; and ‡Department of Pharmacology, Karolinska Institutet, S-104 01 Stockholm, Sweden

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Abstract—The relation between the level of cyclic AMP and bone resorption was studied in a bone organ culture system, using calvaria from newborn mice. Two methylxanthines, iso-butyl-methylxanthine and theophylline and two non-xanthine inhibitors of cyclic AMP phosphodiesterase, Ro 20-1724 and rolipram, stimulated the release of [^{45}Ca] and [^{3}H] from bones prelabelled *in vivo* with [^{45}Ca]- and [^{3}H]proline, respectively. The release occurred after a delay of more than 24 hr. In 120-hr cultures, theophylline, IBMX, rolipram and Ro 20-1724, all stimulated the release of stable calcium, inorganic phosphate and the lysosomal enzymes, β-glucuronidase and β-*N*-acetylglucosaminidase from mouse calvarial bones. In addition, all four phosphodiesterase inhibitors decreased the amount of hydroxyproline in the bones at the end of the culture period. The release of minerals and the decrease of hydroxyproline was abolished by indomethacin. In short-term cultures (24 hr), rolipram and Ro 20-1724 did not reduce PTH-stimulated mineral mobilization, whereas the two methylxanthines, and dibutyryl cyclic AMP and 8-bromo cyclic AMP, did cause a reduction of PTH-stimulated mineral release during the first 24 hr. All four phosphodiesterase inhibitors increased the accumulation of cyclic AMP in the calvaria and inhibited cyclic AMP hydrolysis in extracts of calvarial bone. There was a correlation between the magnitude of the initial rise in cyclic AMP and the delayed stimulation of bone resorption. However, much lower concentrations of the PDE inhibitors were sufficient to produce a delayed increase in bone resorption than to block phosphodiesterase and significantly raise cyclic AMP levels. It is suggested that the elevation of cyclic AMP in a subset of bone cells results in an acute reduction of bone mobilization and the cAMP elevation in another subset to a delayed rise in bone resorption.

Two calcium-regulating peptide hormones, parathyroid hormone (PTH) and calcitonin (CT), both activate adenylate cyclase and elicit a transient accumulation of cyclic AMP in bone cells, despite the fact that they have opposing effects on bone resorption (reviewed in refs. [1, 2]). The reports showing that dibutyryl cyclic AMP can mimic the bone resorption stimulatory effect by PTH [3, 4], as well as the acute inhibitory action of this hormone upon bone collagen synthesis [5], have formed the main basis for the widely accepted concept that cyclic AMP serves as a second intracellular messenger of the action of PTH on bone cells. However, more recent studies have shown that dibutyryl cyclic AMP, cholera toxin and forskolin can, at least transiently, inhibit both spontaneous and stimulated bone resorption *in vitro* [6–10] and these observations have led to the hypothesis that cyclic AMP may be the intracellular messenger for the inhibitory effect on bone resorption caused by CT [9]. These apparently divergent opinions may be partly explained by the finding that dibutyryl cyclic AMP causes a biphasic effect upon mineral mobilization from cultured mouse calvarial bones [11] as well as from fetal rat long bones [12], with an inhibitory phase seen initially and a stimulatory later. When relatively low concentrations of the nucleotide were used it was possible to obtain the delayed stimulatory response on calcium mobilization without a preceding inhibitory phase [11]. The time course of action of PTH is different and a stimulation of bone demineralization can be seen already after 3 hr [13, 14]. We have suggested that the acute stimulatory effect of PTH on bone resorption, which does not involve the recruitment of new osteoclasts, is not mediated by cyclic AMP but that the delayed stimulatory effect of the hormone, coupled to recruitment of new osteoclasts, may be dependent on cyclic AMP [11]. Our view is based on data obtained in neonatal mouse calvaria and fetal rat long bones. However, there are two recent papers reporting a stimulation of calcium efflux from embryonic chick and mouse bones in short-term cultures by forskolin [15, 16], suggesting that in some bone tissues cyclic AMP can rapidly stimulate osteoclasts.

Low doses of the cyclic AMP phosphodiesterase inhibitors aminophylline, theophylline and iso-butylmethylxanthine (IBMX) stimulate basal mineral mobilization from mouse calvaria [6, 11, 17] after a considerable lag phase [11]. When the concentration of these xanthine phosphodiesterase inhibitors was increased, an initial inhibition of basal as well as PTH-stimulated calcium release was found [6, 7], and this is observed also with dibutyryl cyclic AMP [11, 12]. As pointed out by Peck and Klahr [1] the results obtained with xanthine phosphodiesterase

† To whom correspondence should be addressed.

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inhibitors are not conclusive since methylxanthines have other pharmacological actions besides inhibition of cyclic AMP breakdown. To examine further the role of cyclic AMP in the initial, calcitonin-like inhibition and in the delayed, PTH-like stimulation of bone resorption, we have compared the effects of different, structurally unrelated, phosphodiesterase inhibitors upon bone resorption and cyclic AMP metabolism.

MATERIALS AND METHODS

Measurements of bone resorption. Bone resorptive activity of drugs and hormones was mainly analysed by using a bioassay originally described by Reynolds [18]. Calvarial bones from 6–7-day-old mice (CsA), prelabelled with 1.5 μCi [^{45}Ca] four days prior to experimentation, were removed by an aseptic microdissection procedure. The calvaria were divided along the sagittal suture into two halves, which were individually cultured on grids in plastic dishes containing 5.5 ml of CMRL 1066 medium added albumin (0.1% w/v) and modified as described by Lerner and Gustafson [19]. In some experiments the calvarial bones were precultured in indomethacin (1 μM) for 24 hr (10 calvarial halves/10 ml medium). The bones were then washed three times in Tyrode's solution and then further by culturing in basic medium for 3 hr before the explants were transferred to grids and cultured with and without test substances as described above. We have recently shown that this technique improves the response to PTH by 75–100% as compared to the response in bones not precultured in indomethacin [Lerner, submitted for publication].

In most experiments half calvaria from 2–5 different litters were pooled and randomized in different groups according to the experimental protocol. Some experiments were performed in such a way that from one animal one calvarial half was used as a control bone and the other half as an experimental bone (paired half-calvaria). At the end of the culture period the bones were dissolved in 6 M HCl and the radioactivity in an aliquot of the medium and the dissolved bones were analysed with liquid scintillation counting. Mobilization of [^{45}Ca] was expressed as the percent of initial radioactivity. The dose–response curves established are based on 3–6 different experiments in which each concentration of the drugs has been tested at least in quadruplicate. In the kinetic studies, paired calvarial halves from mice prelabelled with 12.5 μCi [^{45}Ca] were cultured as described above and the release of isotope at different time intervals was determined by withdrawal of small amounts of medium (50–250 μl).

The degradation of the organic matrix was assessed by following the release of [^{3}H] from [^{3}H]proline-labelled explants dissected and cultured as described above. The mice were prelabelled with 10 μCi [^{3}H]proline 48 hr before sacrifice. At the end of the culture period the bones were hydrolysed in 6 M HCl at 110°. The hydrolysate was evaporated, redissolved in water and an aliquot of the hydrolysed bones and the media were used for liquid scintillation counting. Corrections for quenching were made using the external standard ratios. Degradation of radioactive proteins was expressed as the percent of initial calculated amounts of [^{3}H]. Using a similar technique, Brand and Raisz [20] found that the release of [^{3}H] ([^{3}H]proline + [^{3}H]hydroxyproline) closely parallels the release of [^{3}H]hydroxyproline, and thus is a good indicator of collagen breakdown.

In separate experiments the remaining amount of hydroxyproline in unlabelled bones was determined at the end of the culture period, as a parameter of bone matrix breakdown. Previously we have found that PTH significantly decreases the amount of hydroxyproline in calvarial bones at the end of a 96-hr culture period [21]. Hydroxyproline in hydrolysed bone samples was assayed by the method described by Kivirikko *et al.* [22]. In these experiments we quantified mineral mobilization by analysing the concentrations of calcium and inorganic phosphate in media before and after culture. Calcium was analysed by atomic absorption spectrophotometry [23] and inorganic phosphate by the method described by Chen *et al.* [24]. In parallel the release of lysosomal enzymes was monitored by assaying the activities of β-glucuronidase and β-*N*-acetyl-glucosaminidase in culture media. The activity of the glycosidases was assayed by measuring the hydrolysis of the appropriate glycoside of phenolphthalein or *p*-nitrophenol according to the methods described by Vaes and Jacques [25].

Determinations of cyclic AMP production. Half-calvarial bones from 5–7-day-old unlabelled mice (CsA) were dissected, pooled and randomized as described above. The bones were then preincubated for 30 min in Ehrlenmeyer flasks containing 2 ml prewarmed and pregassed basic CMRL 1066 medium (four half-calvaria/flask). Subsequently 2 ml medium with and without test substances was added to each flask and the incubation continued for the time periods indicated in the legends to the figures. The incubations were performed in a shaking water bath (40 oscillations/min) at 37°. The reactions were terminated by quickly transferring the bones to 0.7 ml 90% propanol. Cyclic AMP was extracted for 24 hr at room temperature, the calvaria were removed and the extract evaporated [26]. Cyclic AMP was determined following reconstitution of the lyophilized bone samples or in crude media samples by the competitive binding method of Brown *et al.* [27]. More than 80% of the binding material was removed by treatment with cyclic nucleotide phosphodiesterase (Sigma, St. Louis, MO, U.S.A.). Results are expressed as picomoles of cyclic AMP/half calvaria [26].

Assay of phosphodiesterase activity. Phosphodiesterase was extracted from calvarial bones, removed from 5–7-day-old mice, by placing 200 calvaria in 0.1% Triton X-100 (one calvaria/0.2 ml) for 24 hr at 4°. The extract was dialysed at 4° overnight against 400 vol of 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 15 mM mercaptoethanol and 1 mM $MgCl_2$. The dialysis membrane used, Union Carbide (Union Carbide Corporation, Chicago, IL, U.S.A.), with an assigned molecular weight cutoff of 10,000 daltons, was boiled in 0.1 M Na_2CO_3. The phosphodiesterase activity in the retentate was assayed according to Thompson *et al.* [28] using [^{3}H]cyclic AMP as substrate. The product formed by the phosphodiesterase

activity, 5′-AMP, was further converted to adenosine by 5′-nucleotidase from snake venom. The nucleoside was then isolated by ion-exchange chromatography and the enzyme activity was estimated by calculating the percentage conversion of [^{3}H]cyclic AMP to [^{3}H]adenosine. The assays were conducted under conditions where they were linear with respect to time and to the amount of added enzyme. The kinetic studies were performed at starting cyclic AMP concentrations ranging from 0.2 to 128 μM. Drugs to be tested were added together with the enzyme sample and the reaction, which was performed at 30°, was started by addition of substrate (8 μM final concentration). At least seven different concentrations of the phosphodiesterase inhibitors were tested in triplicate to determine IC_{50} values.

Materials. CMRL 1066 medium was prepared from powder obtained from Flow Laboratories, Irvine, Ayrshire, Scotland. [^{45}Ca]$CaCl_2$ (sp. act. 11–44 Ci/g) and [^{3}H]adenosine 3′,5′-cyclic monophosphate (26 Ci/mmol) were from New England Nuclear, Boston, MA, U.S.A. L-[5-^{3}H]proline (37 Ci/mmol) from Radiochemical Centre, Amersham, U.K. Adenosine, adenosine 3′,5′-cyclic monophosphate, 8-bromo guanosine 3′,5′-cyclic monophosphate, dibutyryl adenosine 3′,5′-cyclic monophosphate, 8-bromo adenosine 3′,5′-cyclic monophosphate, theophylline and prostaglandin E_2 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 3-Isobutyl-1-methylxanthine was from Aldrich Chemicals Co., Milwaukee, U.S.A. 4-(3-Cyclo-pentyloxy-4-methoxyphenyl)-2-pyrrolidone (Rolipram = ZK 62711) was a generous gift from Dr Sprzagala of Scherring AG, Berlin, F.R.G. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was kindly provided by Hoffman–La Roche AG, Basel, Switzerland. 8-Phenyl-theophylline was from Calbiochem. Enprofylline (3-propylxanthine) was a gift from AB Draco, Lund, Sweden. Synthetic bovine parathyroid hormone (PTH 1-34) with a potency of 6800 U/mg was obtained from Beckman, Geneva, Switzerland. Synthetic salmon calcitonin with a potency of 5000 U/mg was kindly donated by Sandoz AB, Basel, Switzerland.

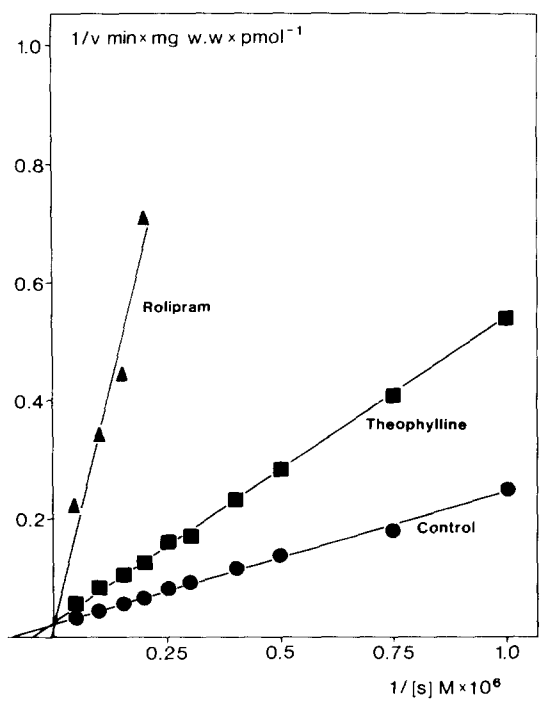

Fig. 1. Double-reciprocal plot of the inhibition of cyclic AMP phosphodiesterase from mouse calvarial bones by theophylline (0.5 mM) and rolipram (0.05 mM). Mean of determinations in triplicate.

RESULTS

Characterization of mouse bone phosphodiesterase

Kinetic studies on the cyclic AMP hydrolysis by mouse bone phosphodiesterase, over a wide range of substrate concentrations (0.2–20 μM), revealed a low K_m form of the enzyme with an apparent K_m of 9.4 μM and a V_{max} of approximately 43.5 pmol/min/mg (Fig. 1). Theophylline (0.5 mM) and rolipram (0.05 mM) increased K_m to 19.6 and 90.9 μM, respectively, with little effect on V_{max}, indicating that the drugs inhibited cyclic AMP phosphodiesterase in a competitive manner. A double reciprocal plot of the data, obtained in assays with substrate concentrations ranging from 20–128 μM, indicated a high K_m form of the enzyme with an apparent K_m of 57 μM and a V_{max} of 202 pmol/min/mg (data not shown).

Effects of theophylline and IBMX on cyclic AMP, phosphodiesterase activity and release of [^{45}Ca]

The two methylxanthines, theophylline (5 mM) and IBMX (0.7 mM), caused a transient increase in cyclic AMP levels in mouse calvaria with a peak value seen after 4 hr of treatment (Figs 2 and 3), followed by a return towards controls, and after 24 hr no difference between treated and control bones was found. The decrease in the cyclic AMP content in the bones could partly, but not totally, be explained by export of the nucleotide from the cells to the incubation medium (data not shown). Residual enzymatic degradation of cyclic AMP may also contribute, as there was a reduction of the total amounts of the nucleotide (medium + bone).

The dose–response curves for the effects of theophylline and IBMX on cyclic AMP response show that these drugs increased cyclic AMP levels at concentrations above 0.3 and 0.01 mM respectively (Figs 2 and 3). Half maximal effect (EC_{50}) was seen at 0.7 mM theophylline and at 0.07 mM IBMX. The cyclic AMP phosphodiesterase activity in homogenates was inhibited in a dose-dependent manner at concentrations of theophylline above 40 μM. Fifty percent inhibition was obtained at 0.3 mM theophylline and at 0.03 mM IBMX.

In short-term cultures (24 hr) theophylline (3 mM) and IBMX (1 mM) as well as dibutyryl cyclic AMP (0.3 mM) and 8-bromo cyclic AMP (0.3 mM) inhibited PTH-stimulated (10 nM) release of [^{45}Ca] (Table 1). In long-term cultures (120 hr) [^{45}Ca] release from mouse calvaria was stimulated by theophylline and IBMX in a concentration dependent way with thresholds at 30 and 3 μM, respectively (Figs 2 and 3). Half maximal stimulation by theophylline was seen at 70 μM and by IBMX at 7 μM.

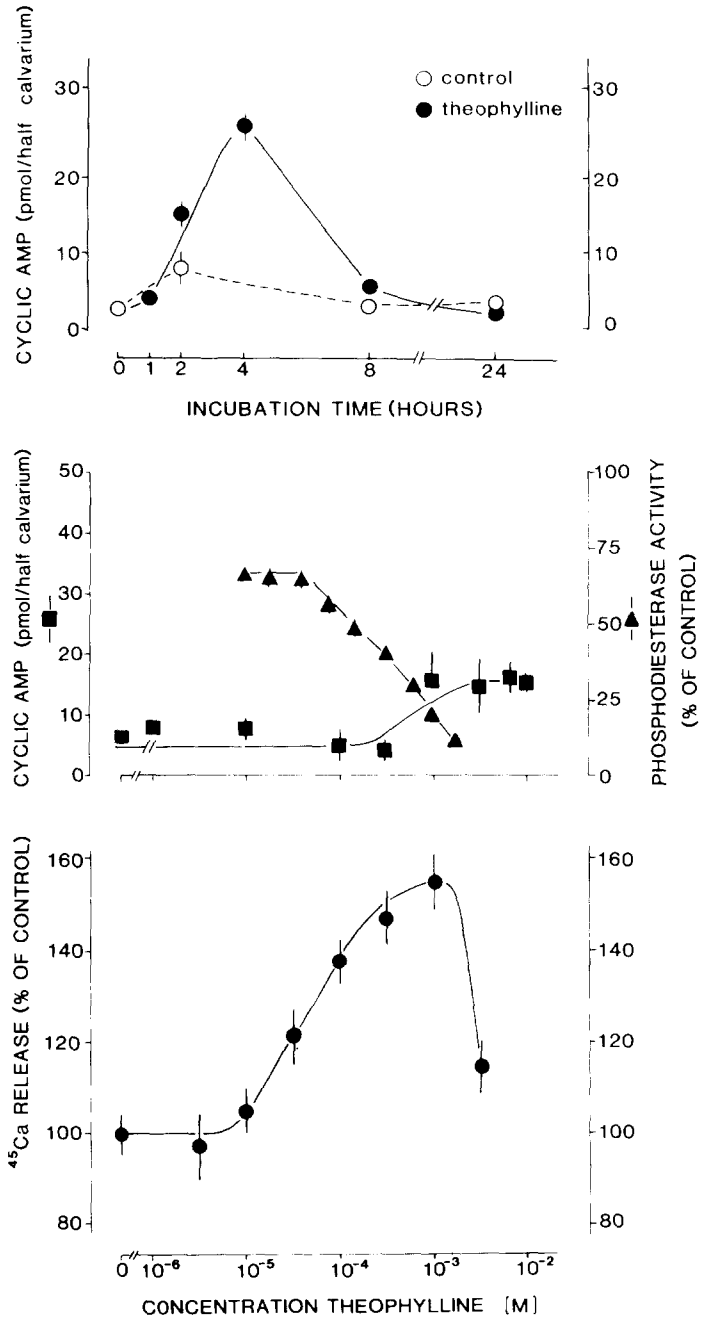

Fig. 2. Studies on the effect of different concentrations of theophylline on tissue cyclic AMP levels and phosphodiesterase activity (middle panel) and on the release of [^{45}Ca] in cultured mouse calvarial bones (bottom panel). In the upper panel is shown the kinetics of changes in tissue cyclic AMP levels after exposure to theophylline (5 mM) for various times. Cyclic AMP levels in the dose–response curve were measured after four hours of incubation and points represent means for four unpaired calvarial halves. Phosphodiesterase activity was assayed with cyclic AMP (8 μM) as substrate and points represent means for triplicate analyses. Release of [^{45}Ca] was analysed after a culture period of 120 hr and points indicate means for 5–15 unpaired calvarial halves. The untreated control bones released 34.0 ± 1.3% (mean ± S.E.M.; N = 14) of the initial [^{45}Ca]. S.E.M.s are given as vertical bars when larger than the radius of the symbol.

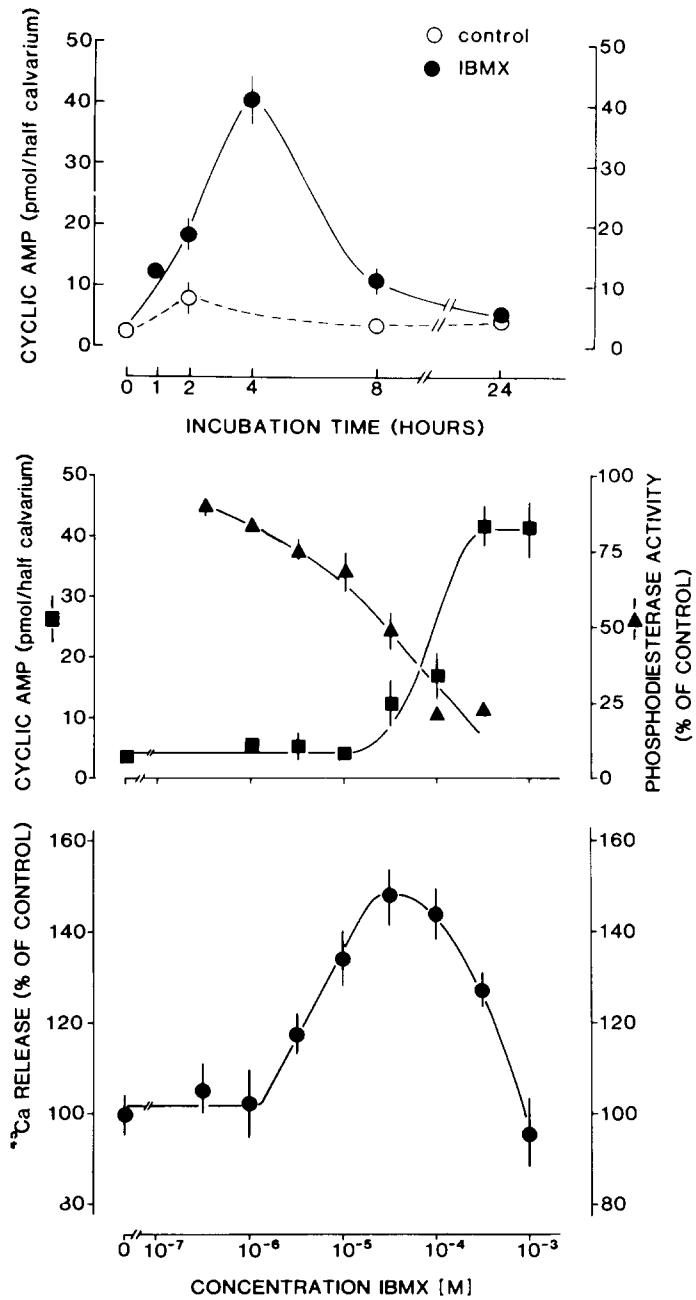

Fig. 3. Studies on the effect of different concentrations of IBMX on tissue cyclic AMP levels and phosphodiesterase activity (middle panel) and on [^{45}Ca] release in mouse calvarial bones (bottom panel). In the upper panel is shown the kinetics of changes in tissue cyclic AMP levels after exposure to IBMX (0.7 mM) for various times. Cyclic AMP levels in the dose–response curve were measured after 4 hr of incubation and points represent means for four unpaired calvarial halves. Phosphodiesterase activity was assayed with cyclic AMP (8 μM) as substrate and points represent means of triplicate assays. Release of [^{45}Ca] was analysed after a culture period of 120 hr and points indicate means for 5–10 unpaired calvarial halves. The untreated control bones released 33.5 ± 2.1% (mean ± S.E.M.; N = 10) of the initial [^{45}Ca]. S.E.M.s are given as vertical bars when larger than the radius of the symbol.

Table 1. Effect of different phosphodiesterase inhibitors on PTH-stimulated release of [^{45}Ca] from mouse calvarial bones

Agent	Amount (M)	% Release [^{45}Ca] Exp. 1*	Exp. 2*	Exp. 3*
—	—	9.3 ± 0.6	7.9 ± 0.3	5.8 ± 0.3
PTH	10^{-8}	12.5 ± 0.3†	15.9 ± 0.7†	15.0 ± 0.9†
+ IBMX	10^{-3}	8.2 ± 0.4‡	11.2 ± 0.9‡	—
+ theophylline	3×10^{-3}	8.0 ± 0.5‡	10.7 ± 0.3‡	—
+ rolipram	10^{-4}	13.6 ± 0.6†	15.4 ± 1.0†	15.3 ± 0.3†
	3×10^{-4}	—	—	14.2 ± 1.8†
+ Ro 20-1724	10^{-4}	13.5 ± 0.5†	16.1 ± 1.3†	17.9 ± 1.9†
	3×10^{-4}	—	—	14.3 ± 1.0†
+ dbcAMP	3×10^{-4}	9.9 ± 0.5‡	10.0 ± 0.5‡	—
+ 8-bromo-cAMP	3×10^{-4}	7.9 ± 0.5‡	9.4 ± 0.6‡	—

Values are means ± S.E.M. for 4–5 unpaired calvarial halves.

* In exp. 1 the bones were directly after dissection cultured for 24 hr in the absence and the presence of PTH, with and without different phosphodiesterase inhibitors. In exp. 2 and 3 the calvaria were preincubated in indomethacin for 24 hr, washed as described in Methods and subsequently prestimulated with PTH for 24 hr. Then medium was changed and the bones were cultured in the absence and the presence of PTH, with and without different phosphodiesterase inhibitor for a third 24-hr period. The [^{45}Ca] release during the last 24 hr was quantified and is given in the table.

† Significantly different from untreated controls ($P < 0.01$).

‡ Significantly different from PTH alone ($P < 0.01$).

However, the dose–response curves were biphasic showing a descending phase at concentrations above 1 and 0.1 mM of theophylline and IBMX, respectively. Addition of indomethacin (1 μM) totally inhibited IBMX (30 μM) and theophylline (0.3 mM) induced stimulation of [^{45}Ca] release in 120-hr cultures (Table 2). IBMX did not stimulate the release of [^{45}Ca] from dead bones (heated in Tyrode's solution at 70° for 5 min; data not shown).

Alkylxanthines, including theophylline and IBMX, have been shown to be many times more potent as competitive inhibitors of adenosine receptors than as inhibitors of phosphodiesterase [29, 30]. It has also been suggested that many of the pharmacological actions of alkylxanthines are due to adenosine antagonism rather than to inhibition of cyclic AMP breakdown [30, 31]. We have therefore used a theophylline-analogue (8-phenyltheophylline) which is a potent blocker of adenosine receptors but a weak inhibitor of phosphodiesterase [29]. This compound, however, did not stimulate [^{45}Ca] mobilization (Table 3). In contrast, enprofylline, a weak inhibitor of adenosine receptors [32] but a potent inhibitor of phosphodiesterase [33], stimulated [^{45}Ca] release in a dose-dependent manner (Table 3).

Table 2. Effect of different phosphodiesterase inhibitors and PTH, in the absence and the presence of indomethacin, on the release of [^{45}Ca] from mouse calvarial bones cultured for 120 hr

Agent	Amount (M)	% Release [^{45}Ca] −indo	+indo
—	—	39.3 ± 2.2	31.3 ± 3.7
IBMX	3×10^{-5}	65.7 ± 2.0*	35.8 ± 1.7
Theophylline	3×10^{-4}	53.9 ± 3.6*	28.1 ± 1.2
Rolipram	3×10^{-5}	62.5 ± 2.6*	31.0 ± 1.1
Ro 20-1724	3×10^{-5}	54.0 ± 3.2*	31.5 ± 1.2
PTH	10^{-8}	63.0 ± 1.7*	66.2 ± 3.7†

Values are means ± S.E.M. for 5–6 unpaired calvarial halves.

* Significantly different from untreated controls ($P < 0.01$).

† Significantly different from indomethacin treated controls ($P < 0.01$).

Effects of rolipram on cyclic AMP, phosphodiesterase activity and release of [^{45}Ca]

Figure 4 shows that rolipram increased cyclic AMP production in mouse bone with maximal stimulation seen after 2 hr. Longer incubation periods resulted in a decrease in the response and after 8 hr no difference in control and experimental bone was seen. The fall in the calvarial cyclic AMP content could be

Table 3. The effect of 8-phenyl-theophylline, enprofylline and theophylline on the release of [^{45}Ca] from murine calvarial bones cultured for 120 hr

Agent	Amount (M)	% release [^{45}Ca]
Exp. 1		
—	—	27.2 ± 1.8
8-Phenyl-theophylline	2×10^{-4}	30.1 ± 1.8
	2×10^{-5}	30.3 ± 2.0
Theophylline	2×10^{-4}	44.9 ± 3.4*
	2×10^{-5}	34.1 ± 3.7
Exp. 2		
—	—	36.9 ± 1.2
Enprofylline	10^{-4}	53.8 ± 2.7
	10^{-5}	45.8 ± 2.6
	10^{-6}	35.4 ± 1.1

Values are means ± S.E.M. for five unpaired calvarial halves.

* Significantly different from control ($P < 0.01$).

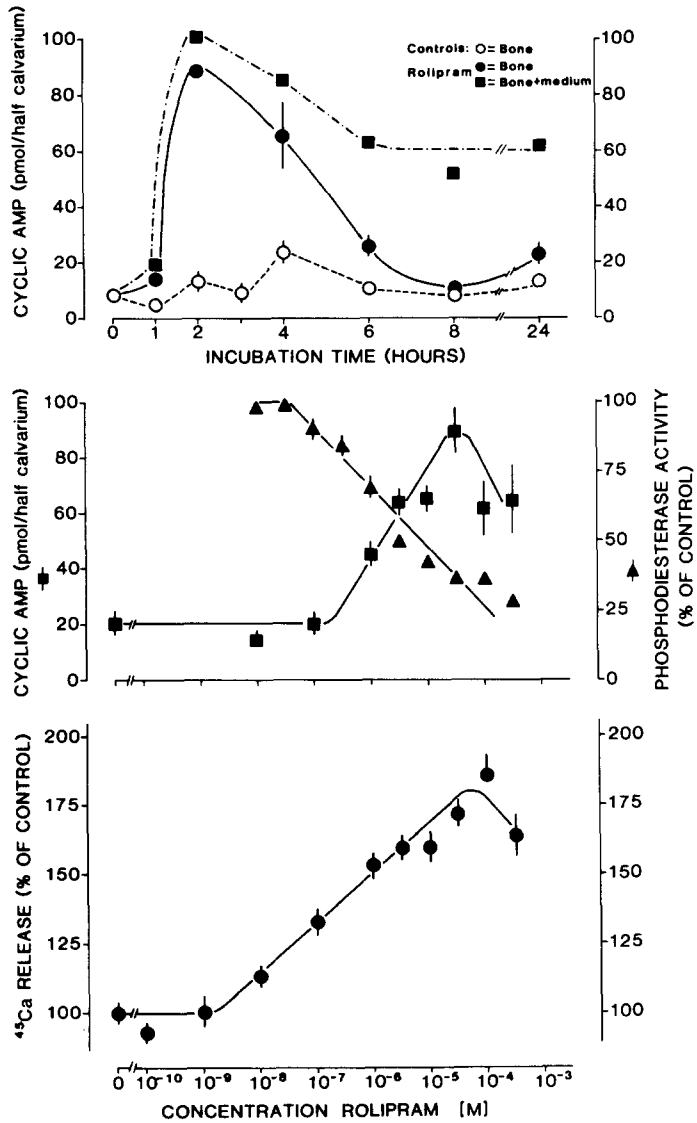

Fig. 4. Studies on the effect of different concentrations of rolipram on tissue cyclic AMP levels, phosphodiesterase activity (middle panel) and on [^{45}Ca] release in mouse calvarial bones (bottom panel). In the upper panel is shown the kinetics of changes in tissue and total (tissue + medium) cyclic AMP levels after exposure to rolipram (0.1 mM) for various times. The dose–response curve for cyclic AMP is based on measurements of nucleotide levels after 2 hr of incubation and points represent means for 3–4 unpaired calvarial halves. Phosphodiesterase activity was assayed with cyclic AMP (8 μM) as substrate and points represent means of triplicate assays. Release of [^{45}Ca] was analysed after 120 hr of culture and points indicate means for 10–24 unpaired calvarial halves. The untreated control bones released 33.1 ± 0.9% (mean ± S.E.M.; N = 24) of the initial [^{45}Ca]. S.E.M.s are given as vertical bars when larger than the radius of the symbol.

partly explained by a loss of the nucleotide from the bones to the media, since the fraction of cyclic AMP in the media dramatically increased after 2 hr of incubation (Fig. 4). The cyclic AMP response to rolipram was higher than the response to theophylline and IBMX. The threshold for increasing cyclic AMP levels was 1 μM and half maximal stimulation was obtained at about 2 μM (Fig. 4). The activity of cyclic AMP phosphodiesterase was inhibited at and above 0.1 μM of the drug and IC_{50} was about 8 μM (Fig. 4).

In long-term cultures (120 hr) rolipram stimulated the spontaneous mobilization of [^{45}Ca] in a concentration-dependent manner with half maximal stimulation at 0.3 μM (Fig. 4). The minimal concentration producing a stimulatory effect was 10 nM. The time-course study of the effect of rolipram (30 μM) on [^{45}Ca] release, presented in Fig. 5, demonstrates that the drug produced its effect on mineral mobilization first after a lag period of at least 24 hr. The effect of calcitonin (CT; 0.1 U/ml) on [^{45}Ca] release stimulated by rolipram (30 μM) was studied in an experiment where the calvaria were pre-stimulated with the drug for 48 hr before addition of CT,

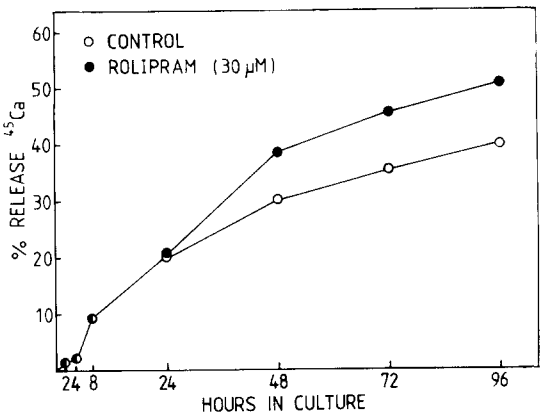

Fig. 5. Time-course study of the effect of rolipram (30 μM) on [^{45}Ca] release from mouse calvarial bones. The release of isotope at stated time intervals was determined after withdrawal of small amounts of medium. Points are means for eight paired calvarial halves and S.E.M.s are given as vertical bars when larger than the radius of the symbol. The stimulatory effect by rolipram was statistically significant at 48, 72 and 96 hr ($P < 0.001$).

to minimize the so-called 'escape from inhibition' [34]. From Fig. 6 it is evident that CT significantly inhibited [^{45}Ca] release stimulated by rolipram with no effect on control release. During the 72-hr treatment period with CT no clear-cut escape from inhibition could be seen. In this experiment all bones were preincubated with indomethacin (1 μM) for 24 hr before exposure to 30 μM rolipram (for details, see Materials and Methods) and the response was much higher (2.4-fold increase of [^{45}Ca] release) as compared to the response in bones directly exposed to 30 μM rolipram (1.5-fold increase; Fig. 4). Also in this experiment the stimulatory effect of rolipram could be registered first after 24 hr of treatment. Stimulation of [^{45}Ca] release in long term cultures by rolipram (10 μM) was abolished by indomethacin (1 μM; Table 2). In contrast to methylxanthines, rolipram (0.1 and 0.3 mM) did not affect PTH-stimulated (10 nM) release of [^{45}Ca] (Table 1). Rolipram did not stimulate the release of [^{45}Ca] from dead bones (data not shown).

Effects of Ro 20-1724 on cyclic AMP, phosphodiesterase activity and [^{45}Ca] release

The cyclic AMP levels in the calvaria were increased after treatment with Ro 20-1724 (Fig. 7). The response peaked at 2 hr and thereafter declined. With this compound the total amounts of cyclic AMP (medium + bone) fall much more than after treatment with the methylxanthines and rolipram. The stimulation of cyclic AMP by Ro 20-1724 was dose-dependent with threshold for action at 3 μM. Half-maximal stimulation could not be stimulated since no plateau-phase was obtained (Fig. 7). The breakdown of [^{3}H]cyclic AMP was inhibited by Ro 20-1724 in a dose-dependent way and with IC_{50} for the inhibition of phosphodiesterase activity at 3 μM (Fig. 7).

The basal release of [^{45}Ca] from mouse calvaria in long-term cultures was stimulated by Ro 20-1724 at and above a concentration of 10 nM and with a half maximal effect seen at 0.3 μM (Fig. 7). Kinetic studies revealed that the effect of Ro 20-1724 (0.01 mM) on spontaneous mineral release could be obtained only after 24 hr of treatment (Fig. 8). As appears from Fig. 8 and Table 1 Ro 20-1724 did not significantly affect the release of [^{45}Ca] stimulated by PTH (0.1 and 0.01 μM), neither in short-term cultures (24 hr) nor in long-term cultures (120 hr). Indomethacin abolished the stimulatory effect by Ro 20-1724 on [^{45}Ca] release in 120-hr cultures (Table 2). No effect on the release of [^{45}Ca] from devitalized

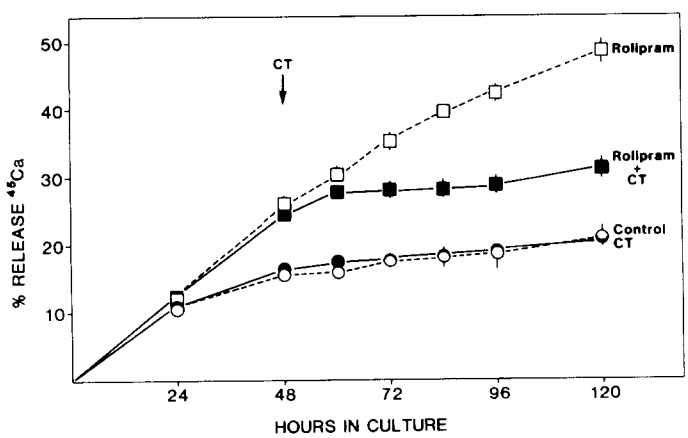

Fig. 6. Effect of salmon calcitonin (CT) on rolipram stimulated release of [^{45}Ca] from mouse calvarial bones. The calvaria were preincubated for 24 hr in indomethacin and washed as described in Methods. Subsequently the calvarial halves were incubated in control medium (○---○, ●---●), or with 30 μM rolipram (□---□, ■---■) starting at zero time on the abscissa. At 48 hr, 0.1 U/ml CT was added to controls (●---●) and to a group preincubated in 30 μM rolipram (■---■). The release of [^{45}Ca] at stated time intervals was determined after withdrawal of small amounts of medium. The points give the means for six unpaired calvarial halves and S.E.M.s are given as vertical bars when larger than the radius of the symbol. The stimulatory effect of rolipram was statistically significant vs controls after 48 hr ($P < 0.001$). The inhibitory effect of CT on rolipram stimulation was statistically significant vs rolipram alone at 72 hr ($P < 0.01$) and at 84, 96 and 120 hr ($P < 0.001$).

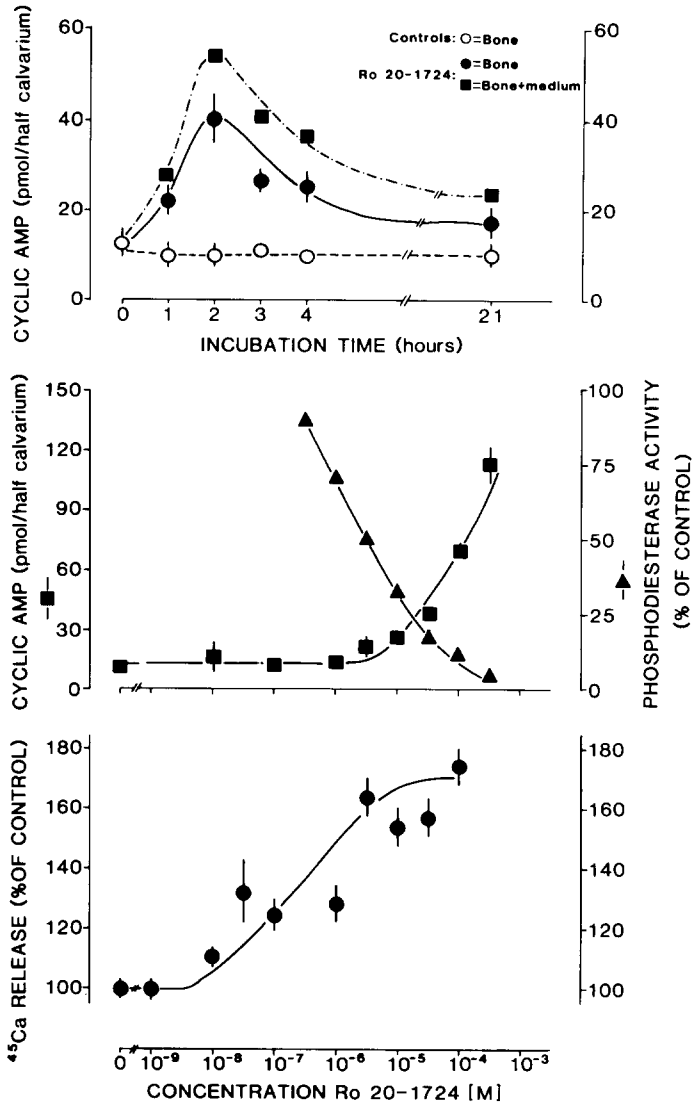

Fig. 7. Studies on the effect of different concentrations of Ro 20-1724 on tissue cyclic AMP levels, phosphodiesterase activity (middle panel) and on [^{45}Ca] release in mouse calvarial bones (bottom panel). In the upper panel is shown the kinetics of the changes in tissue and total (tissue + medium) cyclic AMP levels after exposure to Ro 20-1724 (0.1 mM). The dose–response curve for cyclic AMP is based on measurements of nucleotide levels after 2 hr of incubation and points represent means of 3–4 unpaired calvarial halves. Phosphodiesterase activity was assayed with cyclic AMP (8 μM) as substrate and points represent means of triplicate assays. Release of [^{45}Ca] was analysed after a culture period of 120 hr and points indicate means for 10–18 unpaired calvarial halves. The untreated control bones released 31.4 ± 0.9% (mean ± S.E.M.; N = 18) of the initial [^{45}Ca]. S.E.M.s are given as vertical bars when larger than the radius of the symbol.

bones could be obtained after addition of Ro 20-1724 (data not shown).

Effect of theophylline, IBMX, rolipram and Ro 20-1724 *on the release of* [^{3}H] *from* [^{3}H]*proline labelled bones, hydroxyproline content in bones and lysosomal enzyme release*

In long term cultures (120 hr), theophylline (1 mM), IBMX (30 μM), rolipram (1 μM) and Ro 20-1724 (10 μM) all significantly stimulated the release of [^{3}H] from calvaria prelabelled with [^{3}H]proline *in vivo* (Table 4). IBMX produced an effect that was as large as that obtained by PTH (10 nM; Table 4).

As appears from Table 5 IBMX (30 μM), theophylline (0.3 mM), rolipram (10 μM) and Ro 20-1724 (30 μM) significantly decreased the amount of hydroxyproline remaining in bones cultured for 120 hr. Indomethacin abolished the effect by the phosphodiesterase inhibitors on the decrease of hydroxyproline (data not shown). In addition, IBMX, theophylline, rolipram and Ro 20-1724 significantly stimulated the release of the lysosomal enzyme β-glucuronidase (Table 5). Similar results

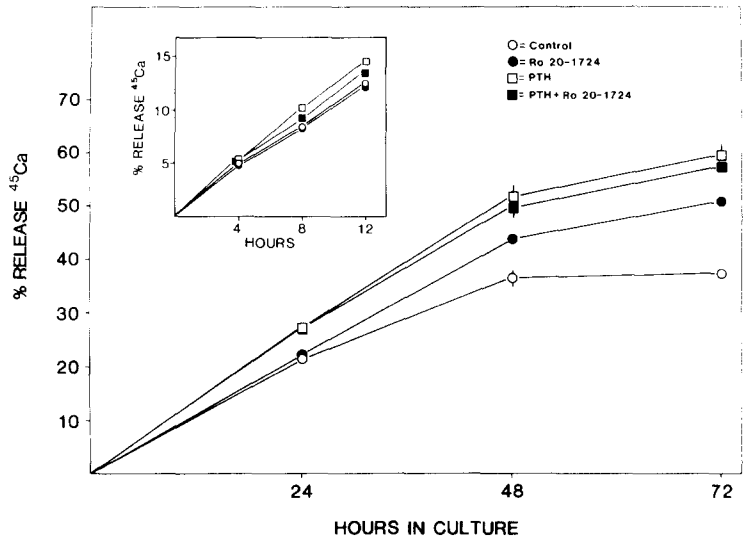

Fig. 8. Time-course study of the effect of Ro 20-1724 (0.1 mM) on spontaneous and PTH-stimulated (0.1 μM) release of [^{45}Ca] from mouse calvarial bones. The release of [^{45}Ca] was analysed after withdrawal of small amounts of culture medium. Points are means for 8–9 unpaired calvarial halves and S.E.M.s are given as vertical bars when larger than the radius of the symbol. The effect of Ro 20-1724 was statistically significant vs controls at 48 hr ($P < 0.05$) and at 72 hr ($P < 0.001$). The effect of PTH was statistically significant vs controls at 8, 24 hr ($P < 0.05$) and at 48, 72 hr ($P < 0.001$).

Table 4. Effect of different phosphodiesterase inhibitors on the spontaneous release of [^{3}H] from mouse calvarial bones prelabelled with [^{3}H]proline

Agent	Amount (M)	% Release [^{3}H]
—	—	32.0 ± 1.6
IBMX	3×10^{-5}	56.4 ± 3.5*
Theophylline	10^{-3}	40.9 ± 2.6†
Rolipram	10^{-6}	42.7 ± 1.2*
Ro 20-1724	10^{-5}	46.3 ± 3.8†
PTH	10^{-8}	56.4 ± 3.4*

Values are means ± S.E.M. for 5–10 unpaired calvarial halves cultured for 120 hr.
* Significantly different from control ($P < 0.001$).
† Significantly different from control ($P < 0.01$).

were seen for β-*N*-acetylglucosaminidase (data not shown). The effect by the phosphodiesterase inhibitors on lysosomal enzyme release was slightly less than that obtained by PTH (Table 5). In these experiments we also observed a significant stimulatory effect by all phosphodiesterase inhibitors on the release of stable calcium and inorganic phosphate and this stimulation was inhibited by indomethacin (1 μM; data not shown).

DISCUSSION

We have compared the effect of four different inhibitors of phosphodiesterase on cyclic AMP accumulation and on the release of radiolabelled calcium in mouse calvarial bones. The phospho-

Table 5. Effect of different phosphodiesterase inhibitors and PTH on hydroxyproline content in bone after culture and on the release of β-glucuronidase in unlabelled bones

Agent	Amount (M)	Hydroxyproline in bone (μg/half calv.)	β-glucuronidase (U × 10^{-5}/half calv.)
—	—	37.4 ± 2.0	5.5 ± 0.5
IBMX	3×10^{-5}	30.8 ± 1.9*	10.2 ± 1.0†
Theophylline	3×10^{-4}	30.3 ± 1.4†	8.3 ± 1.3*
Rolipram	10^{-5}	26.1 ± 0.7†	12.0 ± 1.0†
Ro 20-1724	3×10^{-5}	30.7 ± 1.1†	10.4 ± 1.9*
PTH	10^{-8}	—	15.3 ± 0.9†

Values are means ± S.E.M. for 5–6 unpaired calvarial halves cultured for 120 hr.
* Significantly different from untreated controls ($P < 0.05$).
† Significantly different from untreated controls ($P < 0.01$).

diesterase activity in homogenates of mouse calvarial bones was kinetically inhomogenous. There was evidence for both high and low affinity forms of the enzyme. The apparent substrate affinities for the so-called 'low'- and 'high-K_m'-forms of the cyclic AMP phosphodiesterase are comparable to those reported for the enzyme in rat calvaria [35] as well as in other tissues [36]. No attempt was made to purify the enzyme(s) or to separate the activities into several forms by chromatography. Hence, it is impossible to determine if the kinetic inhomogeneity reflects the presence of several forms of the enzyme. In the studies of the effect of the phosphodiesterase inhibitors, a substrate concentration (8 μM) close to the K_m value for the 'low K_m'-form was used and the contribution to the overall activity by the 'high-K_m' form is likely to be small. The pyrrolidone rolipram and the imidazoline Ro 20-1724 were considerably more potent than the two xanthines IBMX and theophylline as inhibitors of cyclic AMP hydrolysis. The potency of these compounds is in general agreement with previous reports [37].

The addition of one of the phosphodiesterase inhibitors to the bone culture caused a progressive increase in cyclic AMP content. The time course of this increase was much slower than that seen following adenylate cyclase stimulators such as PTH, CT and PGE_2 [38]. Higher concentrations of the phosphodiesterase inhibitor was usually required to obtain a rise in cyclic AMP than to block phosphodiesterase. At least 20% inhibition of the enzyme could be borne without measurable changes in cyclic AMP content. Conversely, an essentially maximal rise in cyclic AMP was obtained before a complete inhibition of phosphodiesterase was obtained. Thus, the dose–response curve for cyclic AMP elevation was much steeper than that for inhibition of phosphodiesterase.

At times after 24 hr all four phosphodiesterase inhibitors caused a clear-cut increase of the release of [^{45}Ca] from prelabelled living bones. This was a cell-mediated process since the drugs failed to increase the release of [^{45}Ca] from dead bones. Moreover, the phosphodiesterase inhibitors stimulated the release of lysosomal enzymes in parallel with increased mineral mobilization, which further indicate a cell-mediated effect [39]. The mineral mobilization probably reflects bone resorption since all the drugs also increased the release of [^{3}H] from bones prelabelled with [^{3}H]proline, and decreased the amount of hydroxyproline in the bones at the end of the culture period, suggesting that the degradation of organic matrix was also stimulated. Furthermore, we noticed that treatment with the drugs resulted in clearly visible large holes in the explants. The finding that CT inhibited [^{45}Ca] release stimulated by rolipram indicates that the activation was dependent on osteoclastic activity.

The rise in cyclic AMP induced by the drugs was generally over within 8–12 hr, but the augmentation of calcium release was not observed until after 24 hr. This is in sharp contrast to the stimulatory effect of PTH on [^{45}Ca] release, which can be observed already after 2–3 hr [13, 14, 21]. The delayed increase of bone resorption induced by the phosphodiesterase inhibitors suggests that cyclic AMP may mediate a late effect of PTH on bone, that is possibly related to recruitment of new osteoclasts, a process requiring about 24 hr [13]. Another explanation may be that the phosphodiesterase inhibitors potentiate the effect of an endogenously produced substance which slowly accumulates in the culture medium.

In tibia from 14-day-old chick embryos Martz and Thomas [15] have reported that forskolin causes a rapid (2 hr) net efflux of calcium from the bones to the culture medium and recently Löwik *et al.* [16] showed that forskolin transiently can stimulate (6–24 hr) calcium release from 18-day-old embryonic mouse calvaria. These observations indicate that cyclic AMP may rapidly activate osteoclasts. However, in neonatal mouse calvaria we have found that forskolin, as well as choleratoxin, causes transient inhibition and only a delayed stimulatory effect on [^{45}Ca] release. Thus, in our system forskolin acts just as the phosphodiesterase inhibitors. A delayed stimulatory effect by forskolin on [^{45}Ca] release has also been found in fetal rat long bones (Lorenzo and Raisz, personal communication). We have no explanation to the contradictory results, but the differences may be related to the fact that different parameters to assess bone resorption were used. Martz and Thomas [15] and Löwik *et al.* [16] have studied net efflux of stable calcium, which represents the net effect of resorption and accretion processes, and we have studied [^{45}Ca] release which more specifically reflects the resorption processes. It should also be noted that, in contrast to PTH, the stimulatory effect of forskolin on calcium release in embryonic mouse calvaria is not associated with any effect on the number of nuclei/osteoclasts and that neither PTH nor forskolin increases the number of osteoclasts/calvaria in these cultures [16].

Our finding that indomethacin inhibited the effect by the phosphodiesterase inhibitors indicates that the drugs stimulated bone resorption by potentiating cyclic AMP formation induced by endogenous prostaglandin E_2. This view is further supported by the fact that in indomethacin treated cultures, IBMX, rolipram and Ro 20-1724 in the presence of forskolin, at low concentrations, stimulate the release of [^{45}Ca] in long-term cultures (Ransjö and Lerner, unpublished). (However, we can not completely rule out the possibility that the phosphodiesterase inhibitors stimulate bone resorption by increasing the biosynthesis of PGE_2.)

The involvement of cyclic AMP in cell growth and differentiation has been extensively studied in many different cell types. Both stimulatory and inhibitory effects on cell division by cyclic AMP have been reported (c.f. [40, 41]). In mouse calvarial bones we have recently found that cyclic AMP analogues and methylxanthines have, depending on drug concentration and culture time, stimulatory as well as inhibitory actions on mitotic activities (Lerner and Hänström, in preparation). However, mitotic inhibitors do not affect bone resorption in long-term cultures of mouse calvaria stimulated by PTH, PGE_2 and dibutyryl cyclic AMP (Lerner and Hänström, in preparation), suggesting that initiation of DNA synthesis is not a prerequisite for the action of these substances on bone resorption. In bone organ culture, however, studies of the recruitment of new

osteoclasts may involve differentiation of precursor cells rather than cell division, a process which may be regulated by cyclic AMP. In other cell types cyclic AMP has been found to affect cell differentiation and in addition, changes in cyclic AMP dependent protein kinase activity during differentiation have been reported in many normal as well as malignant cells (reviewed in [42]).

A significant effect on bone resorption was obtained at concentrations of the phosphodiesterase inhibitors that were 10–100 times lower than those required to produce significant increases in cyclic AMP. Similar shifts in the dose–response curves were obtained also for PTH and PGE_2 (Lerner *et al.*, unpublished). The differences in the dose–response curves could be taken as evidence against a mediator role of cyclic AMP. However, another possibility, which seems more likely, is that only small increases in cyclic AMP—undetectable by our method—are required to stimulate the processes leading to a delayed increase in bone resorption. This is also supported by the finding that an increased activity of cyclic AMP-dependent protein kinase activity can be seen at much lower concentrations of PTH and PGE_2 than those needed to stimulate cyclic AMP accumulation [43].

Previously it has been shown that during the first 24 hr of culture exposure of bone explants to methylxanthines at high concentrations leads to a decrease in spontaneous as well as PTH- and PGE_2-stimulated mineral mobilization [4, 6, 7, 11, 44]. This biphasic time-course explains why the dose–response curves for calcium release, reported in the present paper, decline at high concentrations of theophylline and IBMX. The inhibitory effect of theophylline and IBMX can be further enhanced by adding a non-metabolizable analogue of cyclic AMP [7]. An inhibitory action of IBMX and theophylline as well as by dibutyryl cyclic AMP and 8-bromo cyclic AMP on PTH-induced release of radioactive calcium was also found in the present study. By contrast, the two non-xanthine phosphodiesterase inhibitors, rolipram and Ro 20-1724, did not cause an acute fall in calcium release, nor did the dose–response curves decline at high concentrations. The failure of rolipram and Ro 20-1724 to inhibit PTH-stimulated mineral mobilization in short-term cultures was seen both when the drugs were added simultaneously with PTH and when the phosphodiesterase inhibitors were added to bones prestimulated by PTH. It should be noted that Ro 20-1724, at the concentrations used, caused approximately 90–95% inhibition of cyclic AMP phosphodiesterase. The reason for the differences in the acute effect by the different phosphodiesterase inhibitors is not known. It has been reported that whereas xanthines are able to inhibit both cyclic AMP and cyclic GMP hydrolysis with equal affinity the two other phosphodiesterase inhibitors are relatively selective for cyclic AMP breakdown [37]. It is unlikely that this is the explanation for the present finding since we have been unable to show any effect of cyclic GMP on mineral mobilization (Lerner, unpublished). It is more likely that it is a rise in cyclic AMP that leads to an acute fall in mineral mobilization. This view is further substantiated by our recent finding that the potent adenylate cyclase stimulator forskolin causes a marked fall in mineral mobilization during the first 24 hr [10] and by the finding of Chambers *et al.* [45] that cyclic AMP can inhibit the activity of isolated osteoclasts. The possibility exists that there may be differences in phosphodiesterases in different cell types. For example, an elevation of cyclic AMP in osteoclasts may be associated with a decreased mobilization of bone minerals. If rolipram and Ro 20-1724 are considerably more potent as inhibitors of osteoblast than osteoclast phosphodiesterase an explanation for their failure to inhibit the mobilization of mineral may be found. Such a possibility is compatible with the proposed regulatory role of osteoblasts in bone resorption [46]. In line with this view Shechter [47] recently reported the existence of Ro 20-1724-insensitive but IBMX-sensitive low K_m cyclic AMP phosphodiesterase in bovine heart. Similar results have been obtained in cultured glial cells [48]. However, other possibilities also exist. Xanthines may have effects that are not shared by the two other phosphodiesterase inhibitors. Thus both caffeine and theophylline have an effect on calcium metabolism in muscles [49]. There is also some evidence that enprofylline, which was more active than theophylline on bone resorption, influences intracellular calcium mechanisms [50]. Finally, the difference in ability to produce an initial depression in bone demineralization may be related to the time-course of cyclic AMP elevation. The xanthines tended to produce more longstanding increase than the two other compounds.

Although the present results indicate that there is no correlation between total bone cyclic AMP and the initial reduction of bone resorption after treatment with phosphodiesterase inhibitors, there seems to be a correlation between delayed stimulation of bone resorption by these drugs and total cyclic AMP production. We propose that this action of phosphodiesterase inhibitors is related to an increased cyclic AMP content in osteoblasts or possibly in osteoclast precursor cells.

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