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PROSTAGLANDIN E_2 AND 2-CHLOROADENOSINE ACT IN CONCERT TO STIMULATE BONE RESORPTION IN CULTURED MURINE CALVARIAL BONES

ULF LERNER* and BERTIL B. FREDHOLM†

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

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Abstract—2-Chloroadenosine-induced calcium release from cultured mouse calvarial bones is reduced by inhibitors of prostaglandin production, whereas PTH stimulated calcium release is not. When calvaria were treated with 2-chloroadenosine (10 μM) for 48 hr the production of PGE was significantly increased. The stimulation of PGE synthesis was totally inhibited by indomethacin (1 μM) and partially by hydrocortisone (0.1 μM). When PGE_2 and 2-chloroadenosine, at submaximal concentrations, were simultaneously added to cultures of calvarial bones, in which the endogenous production of prostaglandins was reduced by indomethacin, a supraadditive effect on calcium mobilization by the two agents was seen. No such synergism could be observed between PGE_2 and PTH or between 2-chloroadenosine and PTH. The degree of stimulation in indomethacin-treated bones by 2-chloroadenosine (i.e. when compared to indomethacin-treated controls) was almost the same as that seen in bones stimulated by 2-chloroadenosine in the absence of indomethacin. These data suggest that 2-chloroadenosine can induce bone resorption by a mechanism independent of stimulation of prostaglandin synthesis but that the amount of 2-chloroadenosine stimulated resorption is enhanced by endogenous and exogenous PGE_2.

Adenosine is known to influence the activity of adenylate cyclase in several cell types by occupancy of receptors located on the external side of the plasma membrane [1]. When isolated bone cells or whole calvaria are challenged by adenosine, there is a rapid activation of adenylate cyclase and an increase in the level of cyclic AMP [2–5]. If the bones are exposed to adenosine for longer periods of time, the nucleoside is quantitatively deaminated to inosine and subsequently by purine nucleoside phosphorylase to hypoxanthine [6]. The rapid metabolism of adenosine complicates studies of the effect of this nucleoside on bone resorption and bone formation. In our initial studies of the effect of adenosine on resorption *in vitro*, we have tried to avoid this problem by using 2-chloroadenosine, a non-metabolizable analogue of adenosine [7, 8] which in many tissues has a qualitatively similar effect to that of adenosine [9, 10]. 2-Chloroadenosine stimulates cyclic AMP formation and bone resorption in cultured calvarial bones from newborn mice [8]. This effect can be reduced by agents which inhibit the metabolism of arachidonic acid [11]. This suggests that 2-chloroadenosine-stimulated bone resorption is either directly or indirectly related to the biosynthesis of prostaglandins (or related compounds). We have therefore studied the effect of 2-chloroadenosine on prostaglandin production in mouse calvarial bones and the interaction between 2-chloroadenosine and prostaglandin E_2 (PGE_2) on bone demineralization.

MATERIALS AND METHODS

Synthetic, bovine parathyroid hormone (PTH 1–34) with a potency of 6800 IU/mg was obtained through Beckman (Geneva, Switzerland). The hormone was dissolved in 0.001 N HCl containing 1 mg/ml albumin. PGE_2 was kindly supplied by the Upjohn Co. (Kalamazoo, MI), through the courtesy of Dr. John E. Pike. Indomethacin was kindly provided by Merck, Sharp & Dohme (Haarlem, The Netherlands), and naproxen by Astra (Södertälje, Sweden). 2-Chloroadenosine, hydrocortisone and essentially fatty acid free bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). CMRL 1066 medium was obtained from Flow Laboratories Ltd. (Irvine, Ayrshire, Scotland). $^{45}Ca[CaCl_2]$ (12.1 μCi/g) was obtained from New England Nuclear Chemicals GmbH (F.R.G.). PGE_2, hydrocortisone, indomethacin and naproxen were dissolved in ethanol and then diluted appropriately in culture medium. The final concentration of ethanol did not exceed 0.1%.

Bone resorption bioassay. The technique used for measuring bone resorption activity was mainly based upon the method described by Reynolds, using cultured mouse calvarial bones and quantifying the release of ^{45}Ca from the explant to the medium as a parameter of bone resorption [12]. Two-day-old mice were injected subcutaneously with 1.5 μCi ^{45}Ca. Four days later half calvarial bones were dissected aseptically and washed in Tyrode's solution. Half calvaria from 3–6 different litters were pooled and randomized in different groups according to the experimental protocol. Subsequently the explants were transferred to culture dishes containing 5.5 ml of the chemically defined medium CMRL 1066, modified as described by Lerner and Gustafson [13]. The explants which rested on stainless grids, were incubated at 37° for 48 hr in a gas phase consisting of 5% CO_2 in air. After culture the bones were dissolved in

6 N HCl and an aliquot of the demineralized bone and the culture medium was analysed for radioactivity with a liquid scintillation counter using Aquasol-2 as scintillation liquid. Bone resorption was expressed as the percentage of the total bone isotope (calculated as the sum of radioactivity in bone and media after culture) released into the culture medium.

In one experiment bone resorption was assessed by following the increase of calcium and inorganic phosphate in the medium after culturing calvarial bones for 48 hr. In our hands, the increase of minerals in culture medium, which is the net result of accretion and resorption processes in the bones, parallels the results obtained with the ^{45}Ca-method. Ca^{2+} was analysed with atomic absorption spectrophotometry [14] and P_i by the method described by Chen *et al.* [15].

Analysis of prostaglandin formation. The amounts of prostaglandins in culture media were analysed with a radio-immunoassay method using an antibody to prostaglandin B_1 (PGB_1) obtained from Clinical Assays Inc. (Cambridge, MA). This antibody showed cross-reactivity against PGB_2, PGA_1, PGA_2 of 17, 15 and 1.6% respectively. Prior to assay, PGE_1 and PGE_2 were converted to PGB_1 and PGB_2 respectively, by alkaline treatment. In good agreement with the above mentioned cross-reactivity figures, we found that 1 ng/ml PGE_2 added to basic medium, in which no bones had been cultured, corresponded to 100 pg/ml PGB_1. The coefficient of variation for determinations of PGE in culture medium was 9.2%. Standard curves obtained were almost identical in several independent assays and samples analysed in different assays did not vary more than 15%. Neither indomethacin nor hydrocortisone did interfere with the assays.

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

RESULTS

Indomethacin (1 μM) and naproxen (10 μM) inhibited the stimulatory effect of 2-chloroadenosine on ^{45}Ca release from mouse calvaria as well as the release of ^{45}Ca from control bones, not exposed to 2-chloroadenosine (Table 1). Thus, the percentage increase of ^{45}Ca release induced by 2-chloroadenosine in indomethacin- and naproxen-treated bones were comparable to those seen in bones not treated with these inhibitors of prostaglandin synthesis. By contrast, the stimulatory effect of PTH was uninfluenced by indomethacin and consequently the per cent increase in ^{45}Ca release was enhanced by indomethacin (Table 1).

2-Chloroadenosine (10 μM) stimulated the release of stable calcium and inorganic phosphate and this increase in mineral mobilization was markedly reduced or abolished by indomethacin and hydrocortisone (Table 2). Treatment of calvarial bones with 2-chloroadenosine (10 μM) for 48 hr resulted in significantly increased levels of PGE in culture medium (Table 2). If indomethacin (1 μM) was added together with 2-chloroadenosine (10 μM) the amount of PGE in culture medium was undetectable. Hydrocortisone (0.1 μM) inhibited 2-chloroadenosine (10 μM) stimulated PGE-production by 97%. Similar results were obtained in three independent experiments. 2-Chloroadenosine (4 and 20 μM) produced a dose-dependent stimulation of ^{45}Ca release in indomethacin-treated cultures (Fig. 1). When 2-chloroadenosine at a concentration of 4 μM was added together with PGE_2 (1 or 5 ng/ml; concentrations which by themselves produced only marginal stimulatory effect on calcium mobilization) the stimulation of ^{45}Ca release was greater than the sum of the separate effects (Fig. 1).

A similar supra-additive effect was also seen when 2-chloroadenosine, at a concentration of 20 μM, was added together with low concentrations of PGE_2 (1 or 5 ng/ml; Fig. 1). These concentrations of PGE_2 were in the same range as that found in medium from untreated bones cultured for 48 hr. However, comparisons are difficult to make since there is evidence that calvarial bone cells can degrade PGE_2 [16]. When PGE_2, at concentrations producing a maximal effect on ^{45}Ca release [11], was added together with submaximal concentrations of 2-chloroadenosine, only an additive effect was found (Fig. 1). When PGE_2 and 2-chloroadenosine or PTH were added at maximal effective concentrations, the effect on ^{45}Ca release was lower than that which could be expected from the figures obtained when the agents were added alone (Fig. 1). When 2-chloroadenosine (4 μM) was added together with submaximal concentrations of PTH (0.3 and 1 nM) additive effects on ^{45}Ca release was found (Table 3). However, when

Table 1. Effect of indomethacin and naproxen on the release of ^{45}Ca from mouse calvarial bones stimulated to resorb by 2-chloroadenosine and PTH

Agent	Amount (μM)	% ^{45}Ca-release					
		Control	2-Chloroadenosine	% Stimulation	PTH		% Stimulation
—	—	28.9 ± 1.6	36.2 ± 2.7*	25	38.9 ± 2.6†	2	35
Indomethacin	1	18.8 ± 0.5†	24.7 ± 0.5‡	31	41.3 ± 2.9†		120
Naproxen	10	19.2 ± 0.9†	27.1 ± 0.5‡	41	—		

Values are means ± S.E.M. for 4–5 unpaired calvarial halves. 2-Chloroadenosine and PTH were added to final concentrations of 10^{-5} and 10^{-8} M, respectively.

* Significantly different from untreated controls ($P < 0.05$).

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

‡ Significantly different from 2-chloroadenosine alone ($P < 0.01$).

Table 2. Effect of 2-chloroadenosine in the absence and the presence of indomethacin and hydrocortisone on the release of calcium, inorganic phosphate and prostaglandin E from mouse calvarial bones cultured for 48 hr

Additions	Amount (μM)	Ca^{2+} (μg/half calvarium)	P_i (μg/half calvarium)	PGE (ng/calvarium)
—	—	21.8 ± 1.6	10.3 ± 1.9	2.13 ± 0.2
2-Chloroadenosine	10	31.0 ± 1.8*	25.2 ± 0.8*	24.7 ± 3.8*
2-Chloroadenosine	10			
+indomethacin	1	19.6 ± 3.4†	16.1 ± 0.9†	<0.15
+hydrocortisone	0.1	7.2 ± 2.1†	13.3 ± 0.9†	0.61 ± 0.2†

Values are means ± S.E.M. for 6–8 unpaired calvarial halves.
* Significantly different from control ($P < 0.01$).
† Significantly different from 2-chloroadenosine alone ($P < 0.01$).

2-chloroadenosine at a concentration of 20 μM was added together with PTH (0.3 and 1 nM) the effect on the release of ^{45}Ca was less than additive.

DISCUSSION

We have previously shown that 2-chloroadenosine stimulates bone resorption in cultured mouse calvarial bones [8] and that the 2-chloroadenosine-induced bone resorption in calvaria could be reduced by addition of various inhibitors of prostaglandin biosynthesis, including hydrocortisone and dexamethasone at low concentrations [11]. The basal rate of resorption in these bone explants is also reduced by inhibitors of prostaglandin synthesis [17–19]. By contrast the rate of resorption in bones stimulated by PTH is unchanged by indomethacin [18, present study], or low concentrations of dexamethasone (10 nM) [11]. Furthermore Bockman and Repo [20] found that PTH does not change the amount of PGE_2 produced by rat calvarial bones. These observations suggest that prostaglandins, with bone resorptive capacity [21, 22], could be involved in the mechanism by which 2-chloroadenosine, but not PTH, promotes bone resorption. The data in the present paper show that the biosynthesis of prostaglandins in mouse calvarial bones, as assessed by the amounts of PGs in culture medium capable of binding to an antibody against PGB_1, is enhanced by 2-chloroadenosine. If indomethacin or cortisol was added together with 2-chloroadenosine not only was mineral release reduced but also the amounts of prostaglandins produced. At the concentrations used, cortisol inhibited mineral release more effectively than indomethacin but the reverse was found for prostaglandin production. This suggests that inhibition of arachidonic acid metabolism was not the only mode by which cortisol inhibits 2-chloroadenosine stimulated bone resorption.

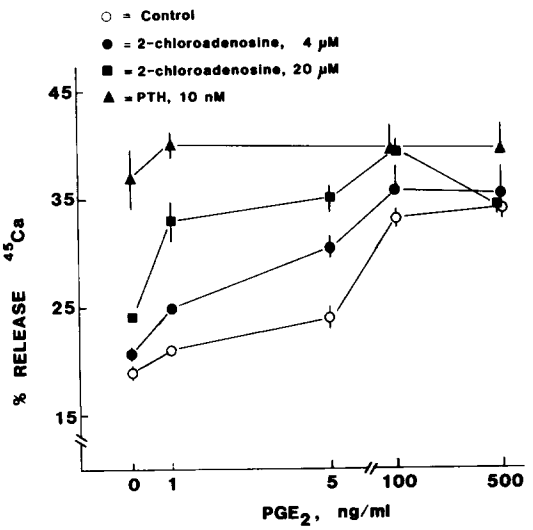

Fig. 1. Effect of 2-chloroadenosine and PTH in the absence and the presence of PGE_2 on the release of ^{45}Ca from mouse calvarial bones treated with indomethacin. In addition to the different stimulators (2-chloroadenosine, PTH, PGE_2) all bones were cultured in the presence of indomethacin (1 μM). Values are means ± S.E.M. for 5–6 unpaired calvarial halves cultured for 48 hr. The effects of PTH, 2-chloroadenosine (4 and 20 μM), in the absence and the presence of PGE_2 were statistically significant ($P < 0.01$). The effect of PGE_2 alone (5, 100, 500 ng/ml) was statistically significant at $P < 0.01$.

It is well known that adenine nucleotides have the ability to stimulate prostaglandin formation [see 23], but to our knowledge stimulation of prostaglandin formation by a nucleoside has not previously been documented. The data in the present paper also suggest that if a certain basal level of prostaglandin production is maintained during culture, a higher rate of resorption in 2-chloroadenosine-stimulated bones can be obtained. Thus low amounts of PGE_2 significantly enhance the stimulatory effects of a suboptimal as well as an optimal concentration of 2-chloroadenosine. When PGE_2 was added in optimally effective concentrations, no potentiation of the stimulatory effect of 2-chloroadenosine on calcium release could be found. The synergistic effect between PGE_2 and 2-chloroadenosine seems to be specific since no such effect could be found between PTH and 2-chloroadenosine. No synergism could be seen between PGE_2 and PTH, probably due to the fact that PTH was present at a concentration which produced a maximal stimulatory effect. However, Raisz *et al.* [24] have previously reported that they were unable to obtain more than additive effects even at suboptimal concentrations of PTH and PGE_2 on ^{45}Ca release from fetal rat long bones. Although the mechanism behind the cooperation between

Table 3. Effect of 2-chloroadenosine, in the absence and the presence of PTH, on the release of ^{45}Ca from mouse calvarial bones

Stimulator	Amount (μM)	PTH (nM)	% Release ^{45}Ca	Δ (treated-control) found	Δ (treated-control) expected
—	—	—	17.5 ± 0.7	—	—
	4	—	21.1 ± 0.5*	3.6	—
	4	0.3	29.8 ± 1.2*	12.3	12.7
2-Chloroadenosine	4	1	38.7 ± 1.9*	21.2	18.7
	20	—	27.7 ± 1.4*	10.2	—
	20	0.3	31.6 ± 0.8*	14.1	19.3
	20	1	31.0 ± 0.8*	13.5	25.3
—	—	0.3	26.6 ± 0.8*	9.1	—
—	—	1	32.6 ± 0.8*	15.1	—

Values are means ± S.E.M. for 6 unpaired calvarial halves. Culture time 48 hr. All calvaria were treated with 1 μM indomethacin.

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

PGE_2 and 2-chloroadenosine remains to be elucidated, it is interesting to note that a similar synergism in bone resorption studies has been demonstrated between endotoxin and PGE_2 [24].

In conclusion the present results suggest an involvement of prostaglandins in 2-chloroadenosine-stimulated bone resorption. On the one hand, 2-chloroadenosine stimulated PG synthesis. On the other hand, PGE_2 and 2-chloroadenosine act synergistically to stimulate bone resorption. However, 2-chloroadenosine is able to stimulate ^{45}Ca release from murine calvarial bones even in the absence of prostaglandins although at a lower rate.

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