2-Chloroadenosine increases calcium mobilization from mouse calvaria in vitro

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Abstract. The effect of 2-chloroadenosine on bone resorption and on cyclic AMP formation in murine calvarial bones in vitro was investigated. 2-Chloroadenosine increased the release of $^{45}$Ca from the cultured bones, but had no effect on dead bones, indicating that the effect is cell mediated. The adenosine analogue remained in the medium for 48 h and caused a transient stimulation of the formation of cyclic AMP. The dose-response curve for the stimulatory effect on cyclic AMP accumulation was linear up to $10^{-4}$M. The dose-response curve for $^{45}$Ca release was linear from $3 \times 10^{-7}$M to $3 \times 10^{-5}$M but then showed a decline in the response. 8-Bromo cyclic AMP inhibited the release of $^{45}$Ca in 24 h cultures. The initial stimulatory effect on bone resorption by 2-chloroadenosine may therefore not depend on cyclic AMP. The level of inosine increased during culture indicating that adenosine is formed by bone tissue.

Adenosine is known to increase the level of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in intact bone and isolated bone cells (Peck et al. 1974, 1975). Adenosine also stimulated adenylate cyclase in plasma membrane enriched fractions from isolated bone cells (Peck et al. 1976). Since it is active in μmol concentrations (Peck et al. 1974), which may be reached locally within the tissue in response to stimuli that enhances ATP utilization or decreases its synthesis (Arch & Newsolme 1978; Fredholm & Sollevi 1981) a regulatory role of the compound in bone cyclic AMP metabolism in vivo could be envisaged.

The role of cyclic AMP in the regulation of bone metabolism is not clear (Peck & Klahr 1979; Lerner 1980a). Despite early evidence suggesting that cyclic AMP serves as an intracellular messenger, of the bone resorptive effect of parathyroid hormone (PTH) and prostaglandins of the E-type (Vaes 1968; Klein & Raisz 1971) the bulk of the current evidence indicates that the initial bone resorption caused by PTH and PGEs is not dependent on cyclic AMP (Herrmann-Erlee & v.d Meer 1974; Peck & Klahr 1979; Lerner & Gustafson 1979; Heersche et al. 1980; Lerner 1980b; McLeod & Raisz 1981). However, late effects of PTH and PGEs may be mediated by cyclic AMP since dibutyryl cyclic AMP and 2 phosphodiesterase inhibitors cause a delayed increase in the rate of bone resorption, possibly due to an increased number of active osteoclasts (Lerner 1980c; Lerner & Gustafson 1981).

It was of interest with this background to see if adenosine, which increases cyclic AMP, would affect bone resorption, and if so, how. To approach this question, we decided to use 2-chloroadenosine rather than adenosine. The reason for this is that adenosine is very rapidly eliminated by cells and tissues in vitro (Arch & Newsholme 1978). The elimination is due to uptake followed by phosphorylation to adenine nucleotides or to deamination by adenosine deaminase. 2-Chloroadenosine is much less susceptible to elimination by either of these pathways (Davies et al. 1980). In the present study we have examined the effect of adenosine and 2-chloroadenosine on bone resorption and on the levels of cyclic AMP.
Materials and Methods

Chemicals were obtained from the following sources: [45Ca]CaCl₂ (11 Ci/g) from Risø, Roskilde, Denmark; Aquasol-2 from New England Nuclear Chemicals GmbH, Dreieich, West Germany; CMRL 1066 medium from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland; essentially fatty acid free bovine serum albumin (fraction V), 2-chloroadenosine and 8-bromo adenosine 3’,5’-cyclic monophosphate (8-bromo cyclic AMP) from Sigma Chemical Co., St. Louis, Mo., USA. 4-(3-cyclo- pentoxy-4-methoxy-phenyl)-2-pyrrolidone (ZK 62.711) was a gift from Dr. Kehr of Scheering AG. 2-Chloroadenosine, 8-bromo cyclic AMP and ZK 62.711 were dissolved directly in culture medium.

Culture technique

Calvarial (frontal and parietal) bones were dissected from 6–7 day-old mice (CsA type) aseptically and washed in Tyrode’s solution. Care was taken not to damage the thin periosteum layer under the dissection procedure. The calvaria were divided along the sagittal suture giving 2 halves, which were separately placed on stainless steel grids in culture dishes containing CMRL 1066 medium, prepared according to the original formula given by Flow Laboratories, but supplemented with 0.1% (w/v) bovine serum albumin, ascorbic acid (150 mg/l) and Fe(NO₃)₂·9 H₂O (100 μg/l). The dishes were put into plexiglass chambers and gassed with 5% CO₂ in air at 37°C (Lerner & Gustafson 1979).

Quantification of bone resorption

The magnitude of bone resorption was assessed by following the release of 45Ca from calvarial halves cultured in 5.5 ml of medium. The bones were prelabelled by injecting the mice with 45Ca sc at least 4 days prior to sacrifice (Reynolds 1976). The radioactivity in the media and bones (after being dissolved in 6 M HCl) was determined with a liquid scintillation counter using Aquasol-2 as scintillant. The release of 45Ca from the bones to the media was determined as the per cent of initial radioactivity (calculated as the sum of radioactivity in bone and media after culture). The experiments were performed with paired half calvaria, i.e. one half (control) was always compared with the other half (experimental) from the same animal. The amount of radioactivity injected in the different experiments is given in legends to the figures.

Determination of cyclic AMP in bone tissue

When cyclic AMP was determined calvarial halves from 3–4 litters were pooled and randomized in different groups. The bones were preincubated for 60 min at 37°C in multiwell dishes (Linbro) containing 2 ml of basic medium without any test substance. The explants were then transferred to medium containing the test substances. The incubations were stopped by quickly placing the bones in 0.7 ml 90% propanol. Cyclic AMP was extracted for 24 h at room temperature. The calvaria were removed and the extract evaporated (Ng et al. 1979). Cyclic AMP was determined following reconstitution of the lyophilized bone samples or in media by the competitive binding method of Brown et al. (1972). More than 80 per cent of the binding material was removed by treatment with cyclic nucleotide phosphodiesterase (Sigma). Results are expressed as picomoles of cyclic AMP/half calvarium.

Determination of purines

The level of purines in the incubates was determined by reversed-phase high performance liquid chromatography, using a Constametric pump, an LDC UV-minotor (254 nm), a Rheodyne injector and a Waters μ-Bondapack C₁₈ column equipped with a 5 cm guard column packed with the same material. The running phase was 0.05 M Na-phosphate pH 6.0 containing 14 per cent methanol (Fredholm & Sollevi 1981). The purines were quantitated by the peak-heights and identified primarily by the retention times. In addition inosine was identified by selective removal by nucleoside phosphorylase (Sigma) treatment.

Results

The effect of adenosine, adenine, 2-chloroadenosine and 8-bromo cyclic AMP on 45Ca release

2-Chloroadenosine, in doses from 10⁻⁶ M to 3 × 10⁻⁵ M caused a dose-dependent increase of 45Ca release in 48 h cultures (Fig. 1). 6 × 10⁻⁵ M had no effect on mineral release and 10⁻⁴ M was inhibitory. The effect of the nucleoside was due to cell-mediated demineralization and not to passive exchange of isotope since 3 × 10⁻⁵ M 2-chloroadenosine had no effect on 45Ca release from dead bones (heated at 70°C in medium for 5 min, data not shown). The stimulatory effect of 10⁻⁵ M 2-chloroadenosine on
Dose-response curve for the effect of 2-chloroadenosine on the release of $^{45}$Ca from murine calvarial bones cultured for 48 h. The bones were prelabelled in vivo by injecting 1.5 $\mu$Ci $^{45}$Ca. The effect is expressed as percentage change of $^{45}$Ca released from a treated bone as compared to its untreated paired control. The control bones released 22.8 ± 0.5% ($\bar{x} \pm$ SEM; n = 47) of the initial radioactivity. Points indicate means and vertical bars SEM for 6–8 paired experiments.

$^{45}$Ca release could be registered 12 h after addition of the test substance and persisted for 96 h (Fig. 2). The addition of adenosine ($10^{-4}$, $10^{-5}$M) or adenine ($10^{-5}$M) did not result in any change of $^{45}$Ca release in 24 h cultures (Table 1). When 8-bromo cyclic AMP ($5 \times 10^{-5}$M) was added to culture medium, a significant decrease of $^{45}$Ca release was seen (Fig. 3). The inhibitory effect of 8-bromo cyclic AMP was observed already after 3 h of culture.
Table 1.
The effect of adenosine, adenine and 2-chloroadenosine on the release of $^{45}$Ca from murine calvarial bones cultured for 24 h.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% release $^{45}$Ca</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>1. Control</td>
<td>18.9 ± 1.2</td>
<td>NS</td>
<td>7</td>
</tr>
<tr>
<td>10$^{-4}$ M adenosine</td>
<td>19.8 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Control</td>
<td>16.8 ± 0.9</td>
<td>NS</td>
<td>8</td>
</tr>
<tr>
<td>10$^{-5}$ M adenosine</td>
<td>16.7 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Control</td>
<td>17.1 ± 0.4</td>
<td>NS</td>
<td>8</td>
</tr>
<tr>
<td>10$^{-5}$ M adenine</td>
<td>16.6 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Control</td>
<td>16.4 ± 0.8</td>
<td>&lt; 0.001</td>
<td>7</td>
</tr>
<tr>
<td>10$^{-5}$ M 2-chloroadenosine</td>
<td>19.0 ± 0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values given are means ± SEM.

The effect of 2-chloroadenosine on cyclic AMP
In agreement with previous results (Peck et al. 1974, 1975) we found that 2-chloroadenosine caused a dose-dependent accumulation of cyclic AMP in the calvarial bones which was linear up to 10$^{-4}$ M (Fig. 4). The time course of the cyclic AMP response to 2-chloroadenosine illustrated in Fig. 5: the level of cyclic AMP peaked after 10 min and

Fig. 3.
The effect of 5 × 10$^{-5}$ M 8-bromo cyclic AMP on the release of $^{45}$Ca from cultured murine calvarial bones. The bones were prelabelled in vivo by injecting 12.5 μCi $^{45}$Ca. Points indicate means from 7 paired experiments and SEM is given as a vertical bar when larger than the symbol's height.

Fig. 4.
Dose-response curve for the effect of 2-chloroadenosine on cyclic AMP levels in murine calvarial bones. Bones were incubated for 15 min in the presence of the phosphodiesterase inhibitor ZK 62.711 (0.1 mM) and different concentrations of 2-chloroadenosine. In addition, one group of bones were incubated in the absence of any test substance (□). Values given are the sum of cyclic AMP in the bones and the media. The level of cyclic AMP in the media in the different groups was on an average 16.0% (range 5.1–26.9) of the total cyclic AMP. Points indicate means and vertical bars SEM for 3–4 calvarial halves.
Table 2.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Inosine (nmol/ml)</th>
<th>Hypoxanthine (nmol/ml)</th>
<th>Adenine (nmol/ml)</th>
<th>2-Cl-ado (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h control</td>
<td>4.7 ± 0.5</td>
<td>11.1 ± 1.3</td>
<td>27.6 ± 1.4</td>
<td>–</td>
</tr>
<tr>
<td>0-time 2-Cl-ado</td>
<td>1.3 ± 0.06</td>
<td>14.9 ± 0.9</td>
<td>26.8 ± 1.3</td>
<td>27.1 ± 1.2</td>
</tr>
<tr>
<td>48 h 2-Cl-ado</td>
<td>5.5 ± 0.7</td>
<td>13.0 ± 2.0</td>
<td>27.6 ± 2.8</td>
<td>23.2 ± 2.2</td>
</tr>
</tbody>
</table>

thereafter declined. After 120 min, the amount of cyclic AMP in the bones was even lower than at zero time. The decrease in cyclic AMP was not due to lack of 2-chloroadenosine, since, as shown in Table 2, 2-chloroadenosine remained in the culture medium for 48 h. The results also show that inosine, the primary metabolite of adenosine, increased 4-fold during 48 h incubation, both in the presence and in the absence of 2-chloroadenosine. When adenosine was added in the concentration 10^{-4} M, more than 70 per cent was metabolized in 24 h. At the same time the concentration of 2-deoxy-adenosine was increased (data not shown).

Discussion

The present results show that 2-chloroadenosine is a potent stimulator of mineral mobilization from cultured calvarial bones. That the increased 45Ca release was due to cellulyarly mediated bone resorption was indicated by the findings that after 48 h of culture large holes in the treated bones could be seen, that no change of 45Ca release from dead bones could be obtained and that exposure of the bones to 2-chloroadenosine also resulted in a loss of stable bone calcium (Lerner & Fredholm, unpublished). Further support to our conclusion that changes in mineral release did reflect bone resorption is gained from our finding that 2-chloroadenosine also stimulated the degradation of bone matrix, as assessed by an increased release of [3H]proline from bones prelabelled in vivo (Lerner & Fredholm, unpublished). The bone resorptive effect of 2-chloroadenosine was manifest already after 12 h and was maintained for at least 96 h. Its potency was at least as high as that obtained with PGE2 in our culture system.

2-Chloroadenosine, in agreement with previous results obtained with adenosine (Peck et al. 1974), stimulated the accumulations of cyclic AMP in bone cells. This effect was maximal after 10 min and was completely over after 2 h. The stimulation was not transient because of degradation of 2-chloroadenosine, since it was well maintained in the medium even during a 48 h incubation. Instead it may be
due to the well-known phenomenon of desensitization or ‘down-regulation’ (Peck & Klahr 1979). Recently Peck & Kohler (1980) demonstrated that, in isolated osteoblast-enriched bone cells, adenosine caused an agonist-specific desensitization and that this nucleoside also made the cells refractory to subsequent stimulation by PTH. It was further found that PTH blunted the subsequent response to adenosine. Thus, it is apparent that adenosine may produce both specific and non-specific desensitization. If 2-chloroadenosine is also capable of producing homologous and/or heterologous down regulation in mouse calvaria, we do not know at present. It should be pointed out, however, that there are reasons to be cautious when comparing results obtained in different systems, since Marcus & Orner (1977) and Heersche et al. (1978) have presented data which indicate that in foetal rat calvaria PTH, PGE_2 and calcitonin (CT) only cause an agonist-specific desensitization, while Herrmann-Erle et al. (1980), also using foetal rat calvaria, have reported that epinephrine and PGE_1 are able to desensitize the bone cell to subsequent PTH challenge.

The dose-response curves for 45Ca release and cyclic AMP formation were not identical. At concentrations above 3 × 10^{-5} M the stimulation of mineral mobilization declined while the increase of cyclic AMP was linear up to 10^{-4} M. Possibly high concentration of 2-chloroadenosine are cytotoxic for the bone cells. Preliminary results indicate that high concentrations of 2-chloroadenosine cause release of lactate dehydrogenase into the medium (Lerner & Fredholm, unpublished). Addition of adenosine to culture medium did not, in contrast to 2-chloroadenosine, result in any stimulation of mineral mobilization. Although this may simply be due to a rapid elimination of adenosine, it has previously been shown that the nucleoside is capable of raising the cellular levels of cyclic AMP (Peck et al. 1974, 1975). This rise in cyclic AMP, however, may not occur in cells that cause bone resorption. Adenosine and CT preferentially increase cyclic AMP in periosteal cells and PTH in subperiosteal cells (Peck et al. 1977). We do not know which cells are equipped with receptors for 2-chloroadenosine coupled to adenylate cyclase, but it seems likely that adenosine and 2-chloroadenosine stimulate the formation of cyclic AMP in the same cells. It is, however, interesting that CT and 2-chloroadenosine seem to stimulate cAMP production in the same population of cells but have opposite effects on bone resorption. As Heersche et al. (1980) have recently suggested that the inhibitory effect of CT may be mediated by cAMP, this could indicate that the cell population used by Peck et al. (1977) were in fact heterogeneous. Alternatively the receptors for CT and 2-chloroadenosine is located on the same cell but the effect of the nucleoside is not mediated by cAMP. Support for such a concept is partly gained from our observations that when cyclic AMP is raised to a certain level both spontaneous and stimulated bone resorption is initially reduced, followed by a delayed stimulation (Lerner & Gustafson 1981). The initial inhibitory capacity of cyclic AMP is also demonstrated in the present paper since 8-bromo cyclic AMP decreased the release of 45Ca. This lipophilic cyclic AMP derivative penetrates the cell membrane and probably acts intracellularly by directly activating cyclic AMP-dependent protein kinases, on which it is more potent than cyclic AMP itself (see Simon et al. 1973).

While our findings provide little information on the mechanism behind the increased bone-resorption caused by 2-chloroadenosine, except that cyclic AMP may not be involved, they raise the possibility that adenosine could be a regulator of bone resorption. In all tissues studied so far adenosine and 2-chloroadenosine have qualitatively similar effects; the latter being generally more potent because of its greater resistance to degradation but also at the receptor 2-chloroadenosine may be slightly more potent than adenosine (Fredholm 1978; Schwabe & Frost 1980). In the present study the threshold for 2-chloroadenosine action was approximately 1 µM. The threshold for the action of adenosine on cyclic AMP is similar (Peck et al. 1974). This threshold concentration is only slightly above the basal adenosine concentration in plasma (Arch & Newsholme 1978; Fredholm & Sollevi 1981), which probably reflects the active concentration in tissues (Fredholm & Sollevi 1981). The concentration may be increased several-fold by a variety of stimuli including hypoxia and increased nerve activity (Arch & Newsholme 1978; Fredholm & Sollevi 1981).

Our finding that inosine, the first breakdown product of adenosine, accumulated in the bone cultures may be taken as evidence that adenosine is formed by bone tissue itself. Studies are in progress to further elucidate the possible role of adenosine in the regulation of bone metabolism and its possible pathophysiological significance.
Acknowledgments

The authors thank Ms. Anita Johansson, Mrs. Ingrid Boström, Ms. Karin Lindgren and Mrs. Britta Lindgren for their skilful technical assistance. The present work was supported by Swedish Medical Research Council (04X-2553, 14X-05426), by the Royal 80 year Fund of Gustaf V, Magnus Bergvalls Foundation, Karolinska Institutet, the Swedish Association Against Rheumatic Diseases and the Swedish Society for Medical Research.

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Received on April 27th, 1981.