The Mode of Action of Dibutyryl Adenosine 3',5'-Monophosphate on Bone Tissue in Vitro*

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SUMMARY

Fetal rat calvaria were incubated in Krebs-Ringer-bicarbonate medium, containing glucose (2 mg per ml) and bovine serum albumin (1 mg per ml). Incubation in a medium with 0.3 or 0.6 mM dibutyryl adenosine 3',5'-monophosphate (DBcAMP) for 15 min caused a 2- to 3-fold increase in the tissue concentration of adenosine 3',5'-monophosphate (3',5'-AMP). This rise could not be explained by degradation of dibutyryl adenosine 3',5'-monophosphate during the incubation period or the isolation procedure since labeled DBcAMP was not altered in the tissue or medium in incubations carried out for either 15 or 60 min. The intracellular concentration of dibutyryl adenosine 3',5'-monophosphate was 0.12 mM after 15 min or 60 min. The intracellular concentration of dibutyryl adenosine 3',5'-monophosphate was 0.12 mM after 15 min of incubation and increased to about 0.26 mM at 2 hours. Dibutyryl adenosine 3',5'-monophosphate was not a substrate for bone cyclic 3',5'-nucleotide phosphodiesterase. Bone phosphodiesterase activity was progressively inhibited by increasing concentrations of dibutyryl adenosine 3',5'-monophosphate which was a more potent inhibitor than theophylline.

The intracellular dibutyryl adenosine 3',5'-monophosphate concentration in the calvaria appears to be high enough to inhibit intracellular phosphodiesterase activity. When 3',5'-[3H]AMP was added to the media, the radioactivity of the calvaria increased continuously for up to 2 hours. After 15 and 60 min of incubation nearly all of the radioactivity was present in the tissue in the form of [3H]adenosine, suggesting either rapid breakdown of 3',5'-[3H]AMP entering the cell or uptake of [3H]adenosine formed extracellularly by breakdown of 3',5'-[3H]AMP. Adenosine could be detected in the media after 60 min of incubation but not after 15 min. These findings indicate that the biological effects of dibutyryl 3',5'-AMP can be accounted for by an increase in the intracellular concentration of endogenous 3',5'-AMP secondary to inhibition of intracellular phosphodiesterase activity.

It has been assumed that N°4-O°dibutyryl-cyclic 3',5'-AMP (DBcAMP) acts intracellularly as an analogue of 3',5'-AMP (1, 2) and that the analogue directly activates the same enzymes controlled by the natural cyclic adeny late. The analogue is usually more effective than the natural compound when added extracellularly and this property has been attributed to more facile entry into cells as well as reduced susceptibility to hydrolysis through catalysis of cyclic nucleotide phosphodiesterase. A recent report, however, showed that cyclic nucleotide phosphodiesterase extracted from the pineal is inhibited by DBcAMP (3).

The findings that 3',5'-AMP is an intermediate in the actions of parathyroid hormone on bone (4-6) and that dibutyryl-cyclic 3',5'-AMP can mimic these actions (7-9) prompted further investigation into the mechanisms accounting for the effect of the dibutyryl analogue. We found that dibutyryl-cyclic 3',5'-AMP added in vitro causes an increase in concentration of endogenous 3',5'-AMP in bone. The use of radioactive dibutyryl-cyclic 3',5'-AMP indicated that there was no significant degradation of DBcAMP to 3',5'-AMP even though intracellular concentrations of the dibutyryl analogue reached 0.2 to 0.3 mM. The latter concentrations were great enough to cause significant inhibition of phosphodiesterase and thereby account for the increase in intracellular concentration of 3',5'-AMP. Some of these observations have been reported in abstract form (10).

EXPERIMENTAL PROCEDURE

Materials—Tritiated dibutyryl-cyclic 3',5'-AMP (N°4-O°dibutyryl-[3°H]adenosine-3',5'-cyclic phosphate; only the adenosine moiety contained tritium, 3.5 Ci per mmole) was obtained from New England Nuclear. Tritiated 3',5'-AMP (16.3 Ci per mmole) was purchased from Schwarz-Mann. Enzymes, nucleotides, and other reagents used in the assay for 3',5'-AMP (11) and phosphodiesterase (5) were obtained and prepared as described previously.

Experimental Conditions—Two milliliters of Krebs-Ringer-bicarbonate (equilibrated with 96% O_2 and 5% CO_2), pH 7.4, each containing glucose (2 mg per ml) and bovine plasma albumin (1 mg per ml) were added to 10-ml flasks. Calvaria were dissected from newborn Sprague-Dawley rats, care being taken that

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the periostea remained intact. The calvaria were cut into equal halves and stored on ice in the incubation medium until use (approximately 30 min). Six calvarium halves (approximately 30 mg, wet weight) were transferred to each flask and were incubated at 37° for 15 min in a Dubnoff metabolic shaker. After incubation they were transferred to cold 0.9% NaCl solution momentarily, then blotted dry on filter paper, and immersed in liquid nitrogen. The calvaria were kept on solid CO₂ until extracted (1 to 2 hours).

Extraction and Assay of 3',5'-AMP—The calvaria from each flask were pulverized in a stainless steel mortar surrounded by solid CO₂. The fine powder was dispersed in 1.5 ml of 90% n-propanol and homogenized in glass with a Tri-R-63 homogenizer equipped with a Teflon pestle. Particulate matter was removed by centrifugation at 12,500 x g for 15 min. The supernatant fluid was lyophilized, redissolved in 50 μl of 90% n-propanol, and chromatographed on Whatman No. 3MM paper with ethanol-0.5 m ammonium acetate (5:2 by volume) as the solvent system. The spot containing 3',5'-AMP was eluted with water and the eluate was applied to a column (0.6 x 3 cm) of Dowex-50 resin (AG 50W-X8, 100 to 200 mesh) in the hydrogen form in order to remove trace amounts of dibutyryl-cyclic 3',5'-AMP and other nucleotides. The column was eluted with water and the 3rd through 7th ml of effluent were collected, lyophilized, and assayed for 3',5'-AMP by the assay method for 3',5'-AMP developed earlier (11). In this assay 3',5'-AMP is converted by purified heart phosphodiesterase to 5'-AMP and the latter is detected by a radioactive phosphate exchange reaction.

Extraction and Assay of Phosphodiesterase—Phosphodiesterase was extracted and assayed with little modification of the method used previously (5). Approximately 500 mg of frozen calvaria were pulverized in a stainless steel mortar surrounded by solid CO₂. The fine powder was suspended in 12 volumes of Tris-HCl buffer (0.05 M, pH 7.4) and homogenized for 30 sec in glass with a Tri-R-63 homogenizer with Teflon pestle. The homogenate was filtered through glass wool and centrifuged at 2200 x g for 15 min. The supernatant fluid was lyophilized, redissolved in 100 μl of 90% n-propanol, and radioactivity measured.

Results

Effect of Dibutyryl-cyclic 3',5'-AMP on 3',5'-AMP Content of Calvaria—Dibutyryl-cyclic 3',5'-AMP, added at concentrations of 0.3 and 0.6 mM, caused a significant increase in concentration of 3',5'-AMP in calvaria incubated for 15 min (Fig. 1). Incubation with dibutyryl-cyclic 3',5'-AMP at 0° did not lead to a rise in concentration of 3',5'-AMP, showing that 3',5'-AMP did not arise from degradation of DBcAMP during the extraction procedure.

Metabolism of Dibutyryl-cyclic 3',5'-AMP—The possibility that the increased 3',5'-AMP concentration in dibutyryl-cyclic 3',5'-AMP-treated calvaria might be caused by degradation of the dibutyryl derivative in the tissue was tested by...
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stock solution DBcAMP

MBcAMP

Phosphodiesterase activity was assayed by measuring the conversion of 3',5'-[3H]AMP and DB[3H]cAMP to 5'-[3H]AMP and dibutyryl-[3H]AMP, respectively. The incubations were carried out as described under "Experimental Procedure." DB[3H]cAMP concentration was 0.6 μM. For control reactions the extract was boiled for 15 min prior to assay for phosphodiesterase.

Purified heart phosphodiesterase activity was determined by analyzing the formation of 5'-AMP from 3',5'-AMP. The reaction mixture contained 1 μl of the phosphodiesterase preparation and 0.06 n mole of 3',5'-AMP in 60 μl of 60 mM Tris-HCl, pH 8.0, containing 1.8 mM MgCl₂ and albumin, 0.1 mg per ml. DBcAMP was added as indicated. After 20 min of incubation at 37° the solutions were boiled for 3 min and then cooled on ice. The 5'-AMP formed was converted enzymatically to ATP and the latter was determined by a radioactive phosphate exchange reaction. Results are the mean ± standard error, based on four determinations.

As shown in Fig. 2, there was no detectable increase after incubation with DB[3H]cAMP in radioactivity in the 3',5'-AMP area (Segments 4 and 5) of the chromatogram. Six-thousandths of the total radioactivity of the starting material was found in this region; after 60 min of incubation five-thousandths of the total activity was found in the 3',5'-AMP segment. If Segment 6 were included there would still be a slight net decrease in the fraction of total radioactivity found in the 3',5'-AMP region. The latter segment is outside the range of migration of 90% of authentic 3',5'-AMP.

Fig. 3. Effect of DBcAMP on cyclic 3',5'-nucleotide phosphodiesterase activity in crude extracts of bone. Phosphodiesterase was assayed by measuring the conversion of 3',5'-[3H]AMP to 5'-AMP as described under "Experimental Procedure," with the exception that the concentration of 3',5'-[3H]AMP was 0.45 μM. Each test mixture contained 200 μg of protein. For control reactions the extract was boiled for 15 min prior to assay for phosphodiesterase. ○—○, no DBcAMP; △—△, 0.12 mM DBcAMP; □—□, 1.2 mM DBcAMP; ■—■, 12 mM DBcAMP. Results represent the mean of duplicate determinations.

Test of DB[3H]cAMP and 3',5'-[3H]AMP as Substrates for 3',5'-Nucleotide Phosphodiesterase from Rat Calvaria

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Incubation time</th>
<th>DB[3H]cAMP</th>
<th>3',5'-[3H]AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>15 min</td>
<td>Not detectable</td>
<td>20.5 ± 1.4</td>
</tr>
<tr>
<td>Crude extract</td>
<td>30 min</td>
<td>Not detectable</td>
<td>37.3 ± 1.3</td>
</tr>
<tr>
<td>Boiled crude extract</td>
<td>30 min</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

Test of Dibutyryl-cyclic 3',5'-[3H]AMP as Possible Substrate for 3',5'-Nucleotide Phosphodiesterase from Rat Calvaria

<table>
<thead>
<tr>
<th>DBcAMP concentration</th>
<th>5'-AMP formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.060 ± 0.005</td>
</tr>
<tr>
<td>0.003</td>
<td>0.061 ± 0.010</td>
</tr>
<tr>
<td>0.03</td>
<td>0.051 ± 0.006</td>
</tr>
<tr>
<td>0.3</td>
<td>0.044 ± 0.000</td>
</tr>
<tr>
<td>3</td>
<td>0.011 ± 0.002</td>
</tr>
<tr>
<td>30</td>
<td>0.002 ± 0.002</td>
</tr>
</tbody>
</table>
Cyclic Nucleotide Phosphodiesterase in Calvaria—Hydrolysis of dibutylryl-cyclic 3',5'-[H]AMP and monobutyrly-cyclic 3',5'-[H]AMP by bone phosphodiesterase was not detectable. 3',5'-[H]AMP was degraded readily, and boiled enzyme showed no detectable activity (Table I).

Inhibition of Bone Phosphodiesterase by Dibutyryl-cyclic 3',5'-AMP—Increasing concentrations of dibutylryl-cyclic 3',5'-AMP caused progressive inhibition of phosphodiesterase activity (Fig. 3). In order to evaluate the possibility that this inhibition merely represented dilution of the cyclic [H]AMP by trace amounts of 3',5'-AMP contaminating the dibutylryl-cyclic 3',5'-AMP preparations the effect of DBcAMP on purified bovine heart phosphodiesterase was determined by the radiochemical assay method for 3',5'-AMP described under “Methods” (11). Increasing concentrations of dibutylryl-cyclic 3',5'-AMP reduced the formation of 5'-AMP, thereby indicating that DBcAMP is a direct inhibitor of phosphodiesterase (Table II).

Comparison of Inhibitory Effects of Theophylline and Dibutylryl-cyclic 3',5'-AMP on Bone Phosphodiesterase—The results of experiments with the crude bone enzyme are shown in Fig. 4. Dibutylryl-cyclic 3',5'-AMP was a more potent inhibitor than theophylline, especially at the lower concentrations used.

Rate of Uptake of 3',5'-[H]AMP or Dibutylryl-cyclic 3',5'-[H]AMP. It was important to determine whether the amount of DBcAMP within the cell reached concentrations sufficient to inhibit phosphodiesterase. In these experiments 3',5'-[H]AMP, dibutylryl-cyclic 3',5'-[H]AMP, or [C]inulin was added to the incubation medium and radioactivity in the calvaria was determined (Fig. 5). The radioactivity of the calvaria incubated with 3',5'-[H]AMP increased continuously during the 2-hour incubation period, whereas calvaria incubated with dibutylryl-cyclic 3',5'-[H]AMP showed a sharp increase in radioactivity during the first 15 min of incubation with little further accumulation thereafter.

[C]inulin uptake appeared to be maximal after 15 to 30 min of incubation. The radioactivity of calvaria incubated with 3',5'-[H]AMP was predominantly adenosine with a small fraction showing the chromatographic mobility of 5'-AMP (Fig. 6). Cyclic 3',5'-AMP could not be detected in significant amounts, a finding which implies that the 3',5'-AMP which entered the intracellular space was rapidly degraded. Adenosine was detected.
in the incubation medium after 60 min of incubation, but not after 15 min (Fig. 7). Theophylline slightly inhibited the uptake of labeled material from medium containing 3',5'-[3H]AMP (0.3 mM, 433,000 cpm per ml) and theophylline (1 mM) were added at the beginning of the incubation. After 1 hour calvaria were removed, washed in cold 0.9% NaCl solution, and blotted dry on filter paper. Radioactivity was extracted by boiling groups of duplicate determinations, and are expressed as ratios of concentrations except as noted.

TABLE III

<table>
<thead>
<tr>
<th>Additions</th>
<th>Medium 3',5'-</th>
<th>Medium 3',5'-</th>
<th>Cabellarum</th>
</tr>
</thead>
</table>
|                    | [3H]AMP     | [3H]AMP     | It is known that dibutyryl-cyclic 3',5'-AMP mimics the effects of a variety of hormones by acting directly as an intracellular substitute for cyclic AMP. The current study indicates that DBcAMP acts indirectly through inhibition of intracellular cyclic nucleotide phosphodiesterase. Inhibition of this enzyme leads to an increase in endogenous 3',5'-AMP within the cell and this increase in endogenous 3',5'-AMP reproduces the biological effects caused by parathyroid hormone interacting at the level of the plasma membrane-adenyl cyclase complex. Recently Klein and Raisz (7) have shown that dibutyryl-cyclic 3',5'-AMP is more effective than theophylline in inducing bone resorption in vitro. Theophylline appeared to be effective only in enhancing the action of doses of parathyroid hormone that were ineffective alone. They found similar dose-response curves for theophylline and DBcAMP and suggested that both compounds acted through a similar mechanism. Our results are compatible with theirs in that we found dibutyryl-cyclic 3',5'-AMP to be more effective than theophylline as an inhibitor of skeletal cyclic nucleotide phosphodiesterase.3

Hydrolysis of dibutyryl-cyclic 3',5'-AMP either in the calvaria or in the incubation media was not detectable in the system described here. The relative amounts of DBcAMP and the mono-
butyryl derivative were also not affected by incubation. This is in agreement with the results of Blecher, Ro'Ane, and Flynn (2) who suggested that dibutyryl-cyclic 3',5'-AMP exerted its action in adipocytes without the removal of either of the two butyryl groups from the nucleotide.

Blecher et al. (2) used dibutyryl-cyclic 3',5'-AMP labeled in the butyryl group and were able to exclude the possibility that as
much as 4% of DBcAMP might be hydrolyzed. This is a significant amount, however. Since intracellular DBcAMP rose to an apparent concentration of 0.2 nM in our experiments, 4% conversion to 3',5'-AMP would increase endogenous concentrations to 0.2 nmoles per g of tissue. The current use of DBcAMP labeled in the adenosine moiety indicated less than 0.1% conversion to 3',5'-AMP which would cause, at most, tissue 3',5'-AMP concentration of 0.2 nM per g. Since intracellular 3',5'-AMP rose to approximately 1 nM per g, the increased concentration cannot be attributed to hydrolysis of dibutyryl-cyclic 3',5'-AMP.

It was mentioned previously by Moore, Iorio, and McManus (15) that equimolar amounts of dibutyryl cyclic 3',5'-AMP did not alter the rate of hydrolysis of 3',5'-AMP by phosphodiesterase. The present work confirms this observation, but also shows a clear-cut inhibitory effect of DBcAMP at higher concentrations.

The effects of parathyroid hormone on bone can be mimicked by DBcAMP but not by 3',5'-AMP (7, 8). Our observations show that there is no significant amount of labeled 3',5'-AMP within calvaria incubated for periods ranging from 15 to 60 min in media containing labeled 3',5'-AMP. This indicates that cyclic 3',5'-AMP is hydrolyzed almost immediately. It seems possible that hydrolysis occurs extracellularly because no labeled 3',5'-AMP was detected in either the cells or the extracellular space at 15 min. Significant amounts of labeled adenosine appear in the media after 60 min, but not after 15 min of incubation. We have found detectable cyclic nucleotide phosphodiesterase activity in the medium, which would account for hydrolysis of some of the 3',5'-AMP in the medium at 60 min. Thus, it seems possible that labeled nucleotides in the cell have entered the cell as 5'-AMP or adenosine. The decreased uptake of labeled nucleotide and the reduced amount of adenosine and 5'-AMP in the media with theophylline support this view.

The intracellular concentration of dibutyryl-cyclic 3',5'-AMP appears to be sufficiently high to account for at least partial inhibition of bone phosphodiesterase, thereby providing further evidence in support of our conclusion that the biological action of DBcAMP on bone is effected predominantly, if not entirely, by increasing the intracellular concentration of 3',5'-AMP secondary to inhibition of intracellular phosphodiesterase activity.

The possibility still exists that part of the effects of dibutyryl-cyclic 3',5'-AMP could be mediated as an intracellular substitute for cyclic AMP. Recent experiments suggest that DBcAMP is active on the muscle phosphorylase kinase (16) or kidney histone kinase systems. However, DBcAMP was much less effective than 3',5'-AMP (by 2 orders of magnitude) and the possibility was not excluded that the samples of DBcAMP used might be contaminated with 3',5'-AMP.

REFERENCES
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