Parathyroid Hormone and Bone Mobilization in Vitro*

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SUMMARY

A two-phase system in vitro has been developed for studying the bone-mobilizing effects of parathyroid hormone. Phase I consists of the incubation of hormone with ascites cells, and Phase II is the incubation of Phase I supernatant with dead bone powder. The mobilization of labeled calcium from this powder is the index of hormonal activity. The response to hormone is apparent at 10^-8 M hormone, appears specific for parathyroid hormone, and has striking analogies to hormone action upon living bone in vivo and in vitro.

One of the well established, direct effects of parathyroid hormone is its ability to bring about the resorption of bone in vivo and in vitro (1-7). Numerous studies have been carried out in an effort to define the mechanism of this effect (8-14). Most of these studies have employed the technique of administering hormone to experimental animals and, at a suitable time thereafter, removing bone to study its metabolizm. Particular emphasis has been placed upon alterations in energy metabolism and associated changes in the production of metabolic acids, particularly citrate and lactate (2, 7-11, 13-17). These metabolic alterations have been produced by grossly impure parathyroid extracts. Recent evidence indicates that there is a peptide, other than the parathyroid hormone, in such extracts which has profound effects in vitro upon glycosylation and Krebs cycle activity (18, 19). Thus, it is not clear whether the reported metabolic changes are important aspects of parathyroid hormone action or are the results of the action of contaminating peptides. This reservation and the lack of a complete correlation between citrate production and bone resorption indicate the need for a careful and cautious appraisal of the acid mobilization theory of Neuman and Neuman (15). Nevertheless, the recent findings of an effect of parathyroid hormone upon ionic fluxes across the mitochondrial membrane (20-24) and the associated changes in oxidative and glycolytic metabolism which these changed fluxes can produce (25-27) make acid mobilization an attractive hypothesis.

It is clear from morphological and biochemical studies (7) that hormone administration leads to destruction of the matrix as well as dissolution of bone mineral. A hormonally induced increase in the collagenase activity of bone may well be an important aspect of hormonal action (28). Likewise, hormone is known to bring about differentiation of bone cells, particularly those cells often associated with bone destruction, the osteoclasts (29, 30). Both of these latter effects mean that hormone probably alters ribonucleic acid and protein synthesis in bone cells; this conclusion finds indirect support from the fact that actinomycin D and puromycin block the mobilization of calcium from bone in parathyroidectomized animals given parathyroid hormone (31, 32). However, it should be made clear that our early studies concerning the effects of actinomycin D indicated an initial elevation of the serum calcium in parathyroidectomized rats treated with hormone (31). This is consistent with the more recent evidence of Talmadge (33) and implies that hormone has a direct effect upon preexisting osteolytic cells, which is not blocked by actinomycin D, and a secondary effect of recruiting more osteolytic cells from nonosteolytic precursors, which is prevented by the prior administration of actinomycin D.

Detailed biochemical exploration of the action of hormone upon bone is made difficult by the low ratio of cellular to noncellular components in this tissue, as well as by the marked heterogeneity of the cell population involved. Some small success has been achieved with the use of bone slices prepared from young animals, but the technical difficulties involved have restricted the usefulness of this type of preparation. Hence, a critical need is that of obtaining a simpler system in which to study this complex phenomenon.

Our approach was predicated upon the assumption that parathyroid hormone influences the metabolism of cells other than those in kidney and bone (21, 34-36). Our aim was to develop, if possible, a system containing a relatively homogeneous population of cells which would bring about hormone-induced bone resorption in a well defined medium. Shortly after the present work began with Ehrlich ascites tumor cells, Borle and Neuman indicated that they had achieved some success in obtaining effects of hormone upon cells grown in tissue culture. This work has been published recently (37) and presents morphological evidence that parathyroid hormone added to cultures of HeLa cells brings about changes in cellular morphology, an increased mitotic activity, an increased rate of cell division, the appearance of multinucleated cells, and, most important of all, the development by these cells of osteolytic activity. Of particular interest was their observation that hormone produced a decrease in cell adhesivenes, indicating the possibility that the hormone altered the properties of the cell membrane. This is of considerable in-

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terest in view of the known effects of hormone upon mitochondrial (20-27) and cell (38) membranes.

The present biochemical results supplement the morphological observations of Borle and Neuman and indicate that a different cell type, ascites tumor cells, can respond to hormone with the production of a "bone-mobilizing" substance, the nature of which remains undefined.

METHODS

Initial studies were carried out by incubating cells, hormone, and bone powder for 4 to 16 hours in a modified Krebs-Ringer-phosphate medium. However, considerable variation in response was observed. It was then found that more consistent results could be obtained if the incubation was separated into two phases: the initial phase, Phase I, the incubation of cells and hormone without bone powder in a modified Krebs-Ringer-phosphate medium; and Phase II, the incubation of bone powder in the supernatant obtained from Phase I.

Phase I—Ehrlich ascites carcinoma cells of the LePage strain were grown in Swiss white mice. They were harvested 5 to 7 days after inoculation. Usually, sufficient cells were obtained from one mouse to carry out 12 to 15 separate incubations. The cells were harvested by low speed centrifugation in a clinical centrifuge and washed three times with a solution containing 120 mM NaCl, 5 mM KCl, 1 × 10⁻⁴ M MgCl₂, and 10 mM sodium phosphate buffer, pH 7.4. Finally, 1 ml of packed cells was suspended in a total volume of 8 ml of the same solution. The incubations were carried out in a Dubnoff shaker maintained at 37°C. The incubation mixture consisted of 0.5 ml of cell suspension added to 2.4 ml of incubation medium containing 120 mM NaCl, 5 mM KCl, 1 × 10⁻⁴ M MgCl₂, and 10 mM sodium phosphate buffer (pH 7.4), and 10 mM glucose. Parathyroid hormone, prepared by the method of Hawker, Glass, and Rasmussen (19), was dissolved in sufficient 0.001 N acetic acid so that the addition of 0.1 ml of solution gave the desired final hormone concentration. A similar amount of 0.001 N acetic acid was added to the control flasks. When other additions were made they replaced an equal volume of medium so that the final volume was 3 ml. After a 1-hour incubation with air as the gas phase, the mixture was chilled, the supernatant was separated from the cells by centrifugation, its pH was adjusted to the desired value with NaOH, and then it was made up to a volume of 3.5 ml with H₂O. In Phase II, 3 ml of this mixture were used.

Phase II—This phase consisted of the incubation of 7 mg of dried powdered bone labeled with ⁴⁶Ca in 3 ml of the supernatant from Phase I for 2 hours at 37°C in a Dubnoff shaker with air as the gas phase. The supernatant was then separated from the bone by filtration through Whatman No. 2 filter paper on a Buchner funnel under vacuum. An aliquot of the filtrate was plated, dried, and counted in an automatic gas flow, end window Geiger counter.

The bone was prepared by subcutaneous injection of a solution containing 20 μC of ⁴⁶Ca into 1-week-old Swiss white mice. They were killed at various times after injection. The parietal bones were collected, boiled for 5 min in deionized water, rinsed, dried overnight in an oven at 100°C, and then ground to a fine powder in a mortar and pestle. The standard bone preparation was obtained from 4-week-old mice, 3 weeks after isotope injection.

Within any single experiment the variation between duplicate samples was ±5%.

Although all effects reported were highly reproducible, the variation between comparable samples from different experiments with different bone or cell preparation was ±15%; hence, a quantitative comparison of results from different experiments was not considered valid. In order to establish the validity of a result, it was necessary to repeat each experiment a number of times. Each result reported in this paper has been demonstrated in three to six separate experiments, each containing a complete set of controls. The results, when comparing separate experiments, showed a much greater variation.

Actinomycin D was obtained from Merck. It was dissolved in 95% ethanol and added to Phase I in a final concentration of 0.1 μg per ml (39). p-Fluorophenylalanine was purchased from Sigma. It was dissolved in water and added in a final concentration of 0.1 mM (40).

RESULTS

If parathyroid hormone was added directly to a suspension of bone powder, there was no increased release of ⁴⁶Ca from the bone powder. However, when the hormone was incubated initially with Ehrlich ascites cells (Phase I) for 1 to 2 hours, it caused the release of some factor into the medium which was...
TABLE I
Effect of peptide hormones on mobilization of \textsuperscript{45}Ca from bone powder

<table>
<thead>
<tr>
<th>Hormone (100 \mu g per ml)</th>
<th>\textsuperscript{45}Ca</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>339</td>
<td>+61.3</td>
</tr>
<tr>
<td>Peak 3</td>
<td>186</td>
<td>-11.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>165</td>
<td>-21.0</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>206</td>
<td>-2.0</td>
</tr>
<tr>
<td>Parotin</td>
<td>207</td>
<td>+27.0</td>
</tr>
</tbody>
</table>

TABLE II
Effect of substrate and oxygen lack upon parathyroid hormone-stimulated release of \textsuperscript{45}Ca from bone powder

<table>
<thead>
<tr>
<th>Condition</th>
<th>\textsuperscript{45}Ca release</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Parathyroid hormone (1 \mu g per ml)</td>
</tr>
<tr>
<td>Glucose (10 mM); air</td>
<td>270</td>
<td>401</td>
</tr>
<tr>
<td>No glucose; air</td>
<td>249</td>
<td>279</td>
</tr>
<tr>
<td>Glucose (10 mM); N\textsubscript{2}</td>
<td>304</td>
<td>294</td>
</tr>
<tr>
<td>No glucose; N\textsubscript{2}</td>
<td>302</td>
<td>241</td>
</tr>
</tbody>
</table>

Fig. 3. The response of a standard Phase II system to Phase I supernatant from hormone-treated cells as a function of the time of Phase I incubation. The results are plotted as the percentage of stimulation above control values obtained with the Phase I supernatant from control flasks. The effect of control Phase I supernatant was not altered with time of incubation. The hormone concentration was 5 \mu g per ml. Specific activity of the bone was 5500 cpm of \textsuperscript{45}Ca per mg.

effective in mobilizing calcium from bone when the supernatant from Phase I cells was incubated with bone powder (Phase II).

The time course of the appearance of \textsuperscript{45}Ca in the Phase II supernatant is shown in Fig. 1. After 2 \frac{1}{2} hours of incubation in Phase II the amount of calcium mobilized by Phase I supernatant from hormone-treated cells was nearly double that mobilized by the control. Calcium mobilization progressively increased for 150 min in the vessels containing Phase I supernatant from hormone-treated cells.

The system was very sensitive to hormone, as shown in Fig. 2. In this case, the percentage of stimulation produced by Phase I supernatant obtained from hormone-treated cells, as compared to controls incubated with bone for 2 hours (Phase II), is plotted as a function of the hormone concentration employed in Phase I. A maximal effect was obtained with 1 to 7 \mu g of hormone per ml, depending upon its purity. Higher concentrations actually led to significant inhibition of the maximal response.

The response was specific for parathyroid hormone, as shown in Table I. Peak 3, a peptide isolated from bovine parathyroid glands (19), stimulated glucose conversion to lactate and CO\textsubscript{2} when added to ascites cells (19), but did not augment calcium mobilization. Parotin stimulated \textsuperscript{45}Ca release by 27\%, and insulin inhibited by 21\%. These effects, although small, cannot be completely attributed to experimental error. The significance of these results is not clear, and further work is in progress to clarify this problem. Note that the concentrations of the other hormones were 100 times greater than that needed for a maximal effect with parathyroid hormone.

The hormonal effect in Phase I required the addition of both glucose and oxygen (Table II). It is not yet known whether other substrates can substitute for glucose. The Phase I reaction is time-dependent (Fig. 3); a maximal effect is obtained between 1\frac{1}{2} and 3 hours. Prolonged incubation resulted in loss of the effect.

Phase II of the system is critically dependent upon the age of the bone and the pH and ionic composition of the medium.

The time between \textsuperscript{45}Ca injection into the mice, used for preparing the bone, and the harvesting of the bone was a critical factor in determining the responsiveness of the system. The specific activity (counts per min of \textsuperscript{45}Ca per mg of bone powder) as a function of time after \textsuperscript{45}Ca injection is plotted in Fig. 4. The maximal specific activity was found between 3 and 4 weeks. This bone proved to be the best for demonstrating the hormonal effect.

As shown in Fig. 5, the pH at which the Phase II incubation was carried out determined the magnitude of the calcium mobilization produced by a constant amount of Phase I supernatant from hormone treated cells. There was a pH optimum at approximately 9.1 when Phase I supernatant from hormone-treated cells was employed, but none with Phase I supernatant from control cells. When the incubation was carried out at this
Actinomycin D (0.1 μg per incubated for 15 min at 80°C. Nevertheless, the prior incubation proteins. Consistent with this observation was an increase in the absorbance at 280 nm observed in the supernatant of hor-
responsible for 45Ca mobilization in Phase II was a protein or material produced in Phase I under the influence of hormone and course of Phase I stimulation raised the possibility that the defining the biochemical basis for hormone-induced bone resorp-
Effect of actinomycin D and p-fluorophenylalanine on parathyroid hormone-stimulated 45Ca release from bone powder

<table>
<thead>
<tr>
<th>Additions</th>
<th>45Ca release</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parathyroid hormone (1 μg per ml)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>247</td>
<td>411</td>
</tr>
<tr>
<td>Actinomycin D (0.1 μg per ml)</td>
<td>243</td>
<td>204</td>
</tr>
<tr>
<td>p-Fluorophenylalanine (0.1 mm)</td>
<td>211</td>
<td>210</td>
</tr>
</tbody>
</table>

pH, the response to Phase I supernatant from hormone treated cells was 4 to 5 times as great as that of Phase I supernatant from control flasks (Fig. 5).
The sharp pH optimum of the Phase II response and the time course of Phase I stimulation raised the possibility that the material produced in Phase I under the influence of hormone and responsible for 45Ca mobilization in Phase II was a protein or proteins. Consistent with this observation was an increase in the absorbance at 280 nm observed in the supernatant of hormone-treated cells when compared to that obtained from control cells. However, the supernatant factor was stable to heat when incubated for 15 min at 80°C. Nevertheless, the prior incubation of cells with either actinomycin D or p-fluorophenylalanine led to complete inhibition of the response of cells to hormone in Phase I (Table III). Neither agent influenced the Phase II reactions when added to Phase I supernatant after hormone had reacted with cells for 30 min or longer. Likewise, hormone added in Phase II without prior incubation with cells in Phase I had no effect upon calcium mobilization.

**DISCUSSION**

The present results, taken in conjunction with those of Borle and Neuman (37), constitute a beginning toward the goal of defining the biochemical basis for hormone-induced bone resorption as well as a start toward the development of a highly specific and sensitive biological assay in vitro for this hormone. It is clear by both morphological and biochemical criteria that HoLa cells and Ehrlich ascites carcinoma cells respond to this agent in a specific manner which leads to the resorption of calcium from dead bone. The dose required is quite small. In our most recent experiments a maximal effect is achieved with 10^{-7} M hormone and a measurable effect with 10^{-8} M. This compares favorably with the dose of hormone employed by Borle and Newman (37) to induce morphological changes, although their changes were observed after a much longer period of incubation than that employed in the present study.

There are many parallels between the known effects of hormone upon living bone in vivo and in vitro (1-14) and those observed upon dead bone in the present study. Also, morphological changes observed by Borle and Neuman (37) are interpretable in terms of those noted with living bone cells (1, 12, 29, 30). The mobilization of calcium from the more mature bone in vitro is predictable on the basis of the known effects in vivo (34). The inhibition of the responses in vitro by actinomycin D and p-fluorophenylalanine (Table III) are similar to effects noted in vivo (31, 32).

Several apparent differences exist, however. Most striking is the fact that the mobilization of calcium in Phase II has a pH optimum at pH 9.1, whereas histochemical evidence suggests that the pH at sites of bone resorption is on the acid side of neutrality (41, 42). No simple explanation of this discrepancy is apparent.

Much of our evidence is consistent with the view that the substance appearing in the supernatant when ascites cells are treated with hormone is a protein or proteins. This possibility is being examined but no definite conclusion is possible.

It is not clear why incubation of cells with hormone in Phase I leads to an initial appearance, and then the subsequent disappearance upon more prolonged incubation, of the bone-mobilizing substance.

The present evidence and that obtained by Borle and Neuman (37) indicate that hormone has no direct effect upon bone dissolution as reported by Gordan (43).

An advantage of the present system over that of Borle and Neuman (37) is the more rapid onset of measurable hormonal changes, the use of completely defined media, and the ability to separate the over-all complex process into two distinct phases. These advantages make possible the study of the effects of hormone upon cellular activity in the absence of bone and the study of the resorptive process in the absence of cells.

It is well to point out that the manner in which our bone was prepared may have an important bearing upon the results obtained. Bone prepared by our method has less 45Ca 2 weeks after injection than at 3 or 4 weeks. Our interpretation of this finding is that the boiling of the bone leads to dissolution of the bone mineral in the readily exchangeable component (partially calcified bone) but not from more mature, calcified, nonexchangeable bone. At 2 weeks there is a higher percentage of the total bone in the exchangeable or partially calcified form, and a greater proportion of 45Ca is in this compartment. Maturation leads to the appearance of a greater percentage of 45Ca in mature, fully calcified bone. Thus, in terms of the system in vitro, the present results imply that hormone mobilizes calcium from mature bone, a conclusion in keeping with kinetic studies in vitro (34, 44). However, it is possible that parathyroid hormone alters the exchange of mineral in the exchangeable compartment of bone in vivo. The present system in vitro is not suitable to test this possibility.
Acknowledgments—We are indebted to Dr. Norman Brink of Merck and Company for the actinomycin D. Miss Berit Johansson rendered expert assistance, and Mr. Charles Hawker was responsible for the preparation of parathyroid hormone used in this work.

REFERENCES

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