On the Hypocalcemic Effect in Rabbits of a Bovine Parotid Extract (PAROTIN)

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Synopsis

The hypocalcemic response in rabbits to parotin (a protein fraction from the bovine parotid gland) was studied in detail. Intravenous parotin produced a significant hypocalcemia appearing at 2 hr, peaking at 4 hr, and returning to the control at 10 hr, after the dosage. The hypocalcemia was distinct in the time course from that induced by thyrocalcitonin or adrenocorticotropin; the latter hormones produced a significant hypocalcemia within 30 min. The evidence was also presented that the parotin effect was neither a consequence of the induced secretion of thyrocalcitonin or adrenocrticotropin, nor that of the suppressed secretion of parathyroid hormone. As judged from the per cent decrease, the extent of hypocalcemia was similar whether the serum calcium was assessed by the assay of total calcium or by the assay of titratable calcium. By contrast, the serum ionic calcium was apparently not influenced by the parotin administration. These results suggest that parotin acts in rabbits, largely independently of the parathyroid and calcitonin system, to cause a decline in the level of serum calcium binding capacity.

In addition to the well-recognized parathyroid hormone, the discovery in the thyro-parathyroid system of a rapidly-acting hypocalcemic principle—calcitonin—enabled one to offer more satisfactory explanations for the constancy of the serum calcium level in mammals (Foster, 1968; Tenenhouse et al., 1968; Aurbach et al., 1969; Copp, 1969; Hirsch and Munson, 1969.) Furthermore, several biological principles, other than parathyroid hormone and calcitonin, have been reported to affect the calcium metabolism. Accordingly, for understanding the mechanisms involved in the calcium homeostasis, it appears of considerable importance to know the similarities and the differences between the effects of these principles and those of the above principal calcium hormones, and to establish, if any, the functional relationship between these humoral factors.

Protein fractions (parotins) have been extracted from the bovine parotid gland that are hypocalcemic in rabbits and effective on some aspects of bone metabolism in rats (Ito, 1954, 1960). Proteinous fractions characterized by the similar biological and chemical properties were also isolated from the human saliva (Ito, 1954, 1960). Although some physiological significances were implied to these extracts by Ito and others, much apparently remains to be solved.

This report is concerned with the detailed studies on the hypocalcemic response produced by a bovine parotid extract (parotin) in rabbits. Comparison is also attempted with the hypocalcemic responses induced by other factors. It is concluded that the parotid extract, administered intravenously, causes a hypocalcemic response which is distinct from that induced either by calcitonin or by

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adrenocorticotropin.

**Materials and Methods**

A protein fraction was extracted from the bovine parotid glands and purified according to the procedure (Ito, 1960) developed by Ito and others for the isolation of “Parotin” (fraction, MP-1, hereafter referred to as MP-1). This fraction was biologically (as judged from the hypocalcemic and leukopenic effects in rabbits) as potent as reported for the authentic parotin. The other hormones were from commercial sources: parathyroid hormone (Para-thormone, Lilly), calcitonin (porcine thyrocalcitonin, Wilson), adrenocorticotropin (Cortrophine, Daiichi), and synthetic β1-24 adrenocorticotropin (Organon).

Male white rabbits of 2.5 to 3.0 Kg body weight were maintained on a rabbit chow (Oriental, Tokyo). Some of them were surgically thyroidectomized one month prior to the use. In this maneuver, one pair of the extrathyroidal parathyroid glands were left unharmed. The animals were employed for the experiment after a 24 hr fasting. Either MP-1, hormones or the respective vehicles were administered via the marginal ear vein. Blood samples were obtained from the different ear vein as needed. Serum was separated within one hr after bleeding.

Calcium in serum was assayed by either of the following methods depending on the purpose of particular experiments. When ample amounts of sera were available, total calcium was measured by atomic absorption spectrophotometry. When the specimens were limited in volume, total calcium was bioassayed by the use of a stoichiometric respiratory burst in isolated rat-liver mitochondria that is induced by the added calcium (Rasmussen *et al*., 1965). Mitochondria were isolated from male Wistar rats according to Rasmussen and Ogata (1966). The respiratory activity was measured polarographically with a Clark type electrode. The respiratory burst induced by a sample calcium was measured in a reaction medium described in the legend of Fig. 1 and quantitated by comparing it with that induced by prior addition of a standardized dose of ADP; the amount of oxygen consumed during the respiratory burst by calcium is half of that of the respiratory burst induced by equimolar ADP (Chance, 1965). By this method 50 μl of the serum of the calcium concentration of 1.6 mM to 6.4 mM gave a satisfactorily linear dose-response (Fig. 1). Calcium ion activity was measured with a calcium-ion-sensitive electrode (Orion). Titratable calcium was measured by an EDTA-titration method (Bachea *et al*., 1958). Free fatty acid was measured according to Dole (1956).

**Results**

1. **Time-course of hypocalcemia induced**
by parotid extract

As already described by Hiramatsu and others (1964), sequential withdrawals from a single rabbit of such amounts of blood as were needed for the conventional calcium assay, caused hypocalcemic responses not infrequently. This is considered to be a consequence of significant blood loss. We therefore developed the mitochondria method for the calcium assay that requires the blood specimen of less than 0.5 ml at each time point. In a preliminary experiment, it was shown that 5 to 7 withdrawals of this amount of blood did not seriously disturb the serum calcium level, and that no significant diurnal variation took place in the meantime. As shown in Figure 2, an intravenous administration of MP-1 (2 mg per Kg body weight, in 2 ml of saline) induced a significant hypocalcemia appearing at 2 hr, peaking at 4 hr, and returning to the control level at 10 hr after the injection. It was also noted that the maximal hypocalcemia as expressed by the per cent decrease (10–15%) was almost identical, whether the serum calcium was measured by total calcium (atomic absorption spectrophotometry, or mitochondria method) or by titratable calcium.

2. Parotid-extract induced hypocalcemia, and calcitonin and adrenocorticotropin induced hypocalcemia

Either porcine thyroid calcitonin (0.2 MRC U per Kg body weight) or synthetic adrenocorticotropin (125 μg per Kg body weight) induced a hypocalcemic response which, in sharp contrast to that of MP-1, appeared within 30 min (Fig. 3). This indicates that
MP-1 is different in its mode of hypocalcemic action from exogenous calcitonin and from exogenous adrenocorticotropin. However, the possibility can not be excluded that the MP-1 effect develops via the secretion of endogenous calcitonin or adrenocorticotropin. Therefore this possibility was examined in the following experiments.

3. Parotid-extract induced hypocalcemia in thyroidectomized rabbits

Though the thymus and parathyroid gland may participate to some extent in the calcitonin secretion, the thyroid is considered to be the major source of calcitonin in most mammals (Hirsch and Munson, 1969.) Thus, the MP-1 effect was examined in thyroidectomized rabbits to test the role of thyroid (therefore, thyrocalcitonin) in the MP-1 induced hypocalcemia. As depicted in Figure 4, the thyroidectomized animals responded to the parotid extract with a hypocalcemia that was indistinguishable in the extent and in the time-course from that produced in the intact animals. Accordingly, it is concluded that the MP-1 induced hypocalcemia is not mediated by the thyrocalcitonin.

4. Serum free fatty acids and calcium levels and effect of parotid-extract

As shown above and also reported from other laboratories (Natelson et al., 1963),
adrenocorticotropin and some other extracts from the pituitary are hypocalcemic in rabbits. Then, the report indicating a parotin-induced stimulation of the adrenocorticotropin secretion (Ito, 1954) leads to a suggestion that the hypocalcemic response is secondary to the effect on the pituitary. Since this possibility cannot be analyzed at present by directly measuring the adrenocorticotropin secretion in vivo, and since the pituitary extracts, besides lowering the serum calcium, elevate the serum levels of free fatty acids in rabbits (Friesen, 1964), the possibility was tested by following the serum free fatty acids levels.

That a dose of crude adrenocorticotropin extract from the bovine pituitary (Cortropine, Daiichi) causes in rabbits a marked elevation in the serum free fatty acids concomitant with the depression of the serum calcium, was confirmed by experiments illustrated in Figure 5. On the contrary, the hypocalcemic response to MP-1 was not accompanied by such elevation in the serum free fatty acids levels. These results suggest, though they do not conclude, that there is no likelihood that the MP-1 effect develops through activation of the adrenocorticotropin system.

5. Parotid-extract induced hypocalcemia in rabbits treated with parathyroid hormone

Another mechanism that is possible as the basis of the MP-1 induced hypocalcemia may be the inhibition of hormone secretion from the parathyroid glands. This might be tested most adequately by examining the MP-1 effect in the parathyroidectomized rabbits. However, owing to the complexity in the anatomical situation of the parathyroid glands in rabbits, a reliable preparation of the parathyroidectomized animals could not be
Fig. 5. Comparison of effects of parotid extract and those of a crude adrenocorticotropin extract on the serum levels of calcium and free fatty acids. Either MP-1 (2 mg/kg body weight) or a crude corticotropin extract from the bovine pituitary (40 ACTH units) was injected into rabbits. Blood samples were collected 4 hr or 1 hr after the injection of MP-1 or adrenocorticotropin, respectively. The calcium was assayed by the titration method. The data are presented as the mean (column) ± S. E. (bar) with the number of experiments (in parentheses). Synthetic β1–24 adrenocorticotropin, in a comparable dose (40 ACTH units), produced a similar enhancement of the serum free fatty acids levels.

obtained constantly by our hands. As an alternative, we examined the MP-1 effect in the thyroidectomized rabbits that were concurrently treated with 100 USP units of parathyroid hormone (Table 1). Because the exogenous parathyroid hormone caused a significant elevation in the serum calcium level, it is assumed that the endogenous hormone secretion from the parathyroid glands is practically abolished. The commitment of thyrocalcitonin was excluded in this experiment by employing the thyroidectomized animals. Such an experimental setting may offer a situation where the MP-1 effect can be tested under the influence of the same dose of exogenous parathyroid hormone. The data of Table 1 clearly show that MP-1 produces the similar extent of hypocalcemia in the animals, irrespective of whether or not they are treated with the exogenous parathyroid hormone. Therefore, the inhibition of the parathyroid hormone secretion may not account for the MP-1 induced hypocalcemia.

<table>
<thead>
<tr>
<th>State of animals</th>
<th>Treatment</th>
<th>mg calcium per 100 ml serum</th>
<th>Number of animals</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a intact</td>
<td>vehicle</td>
<td>12.7±0.5**</td>
<td>7</td>
<td>P&lt;0.01***</td>
</tr>
<tr>
<td></td>
<td>MP-1</td>
<td>11.0±0.3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 mg/Kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b thyroidectomy</td>
<td>vehicle</td>
<td>13.4±0.2</td>
<td>7</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>MP-1</td>
<td>12.3±0.2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 mg/Kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTH 100 USP*</td>
<td>13.9±0.1</td>
<td>7</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>PTH + MP-1</td>
<td>12.9±0.4</td>
<td>7</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Blood samples were obtained 4 and 7 hr after the administration of the vehicle or the reagents. The calcium was estimated by the titration method. The table contains the results of 7 hr samples. Similar trends are obtained in the results of 4 hr sample.

*parathyroid hormone
**mean ± S.E.
***difference from the vehicle

Table 1. Effects of MP-1 and parathyroid hormone on serum calcium levels

6. Effect of parotid-extract on serum calcium ion activity

In view of the currently prevailing view that the function of the two major calcium hormones, parathyroid hormone and cal-
Table 2. Effects of MP-1 on serum calcium fraction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals</th>
<th>mg calcium per 100 ml</th>
<th>Total Ca</th>
<th>Ca++</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>7</td>
<td>12.7±0.5*</td>
<td>5.2±0.2*</td>
<td></td>
</tr>
<tr>
<td>MP-1 2 mg/Kg</td>
<td>7</td>
<td>11.0±0.3</td>
<td>5.2±0.1</td>
<td></td>
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</table>

The blood samples were collected 7 hr after the administration of MP-1 or the vehicle. The serum calcium concentration and calcium ion activity were measured by the use of the atomic absorption spectrophotometry and a calcium ion sensitive electrode (Orion, model 92-20), respectively. Similar results, viz. significant fall in the total calcium level without change in the ionic activity, were also obtained with the samples of 4 or 5 1/2 hr after the MP-1 treatment. *mean ±S.E.

calcitonin, is intimately correlated with the calcium ion activity in the circulation (Copp, 1969), the effect of MP-1 on this parameter of the calcium metabolism was studied. The data of Table 2 disclosed that MP-1, though it significantly depresses the serum concentration of total calcium, does not affect the calcium ion activity.

Discussion

The present study clearly indicates that the hypocalcemic response of rabbits to parotin is unique in that; first, the time course is distinct from that of the hypocalcemia induced by thyrocalcitonin or adrenocorticotropin; second, the hypocalcemia is not associated with a significant fall in the ionic calcium; and third, it is not a consequence of either the induced secretion of thyrocalcitonin and adrenocorticotropin or the suppressed secretion of parathyroid hormone. Thus, in terms of serum calcium metabolism in rabbits, the parotid system appears to function independently of the well-established parathyroid and calcitonin system. In view of a large body of evidence that in mammals the parathyroid and calcitonin system plays the supreme role in the maintenance of stability of serum calcium ion activity (Copp, 1969), any modest influence on the serum ionic calcium may be counterbalanced by the action of either parathyroid or calcitonin. The hypocalcemia by parotin was modest in the extent and developed gradually after a latent period of more than 2 hr. This ample time span appears sufficient for the parathyroid system to counteract the decline, if it occurred, of the serum ionic calcium. Thus, it seems premature to exclude the possibility that parotin affects the level of serum ionic calcium, before the pertinent experiments, for example, experiments in parathyroidectomized rabbits, confirm this concept. Furthermore, we have obtained evidence in rats that parotin possesses, in addition to its hypocalcemic effect, such property as to potentiate the hypocalcemic action of exogenous calcitonin (Shimazawa et al., 1969). Considering the definite effect of calcitonin on the serum ionic calcium, this suggests that parotin may somehow influence the level of serum ionic calcium. At any rate, the present results indicate that this aspect of parotin action is of such limited extent as to be counterbalanced by the potent endogenous parathyroid system. This is in concert with our observations in rabbits and rats that the hypocalcemia by parotin has never been associated with tetany-like signs or changes in the Q-T segment of the electrocardiograms.

Lack of the fall in the ionic calcium in the face of considerable decrease in both total and titratable calcium implies that the major consequence of the parotin action is a diminished calcium binding capacity of serum protein. This was supported partly by our preliminary experiments that disclosed a moderate decrease (approximately 10%, on the average) in the serum total protein coinciding with the hypocalcemia. However, if one assumes that one g of serum protein binds 0.8 mg of calcium (Rawson and Sunderman, 1948), the above extent of the fall in the total serum protein (decrease in total protein by 0.5–1.0
g/100 ml) does not account for all of the decline in the serum calcium level (decrease in total calcium by 1–2 mg/100 ml). Therefore, the possibility cannot be excluded that parotin induces a change in the qualitative as well as quantitative aspects of serum protein. In this context, the recent reports from the laboratory of Aonuma and others (1970, a and b) are of some interest. They presented evidence that in rodents parotin induces a pituitary stimulation of the testis resulting in the enhanced secretion of androgens. Androgens are shown in our laboratory to influence the serum protein as well as to sensitize animals to the action of calcitonin (Ogata et al., 1970); the effects which are shared by parotin. The concept that the parotin effect on the testis has some bearing on the hypocalcemic response is currently under study in our laboratory.

References