INHIBITION BY CHOLERA TOXIN OF PARATHYROID HORMONE-INDUCED CALCIUM RELEASE FROM BONE IN CULTURE

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Received August 11,1977

SUMMARY

Cholera toxin was added to the culture of fetal rat limb bone and its effect on calcium release as well as on adenylate cyclase activity was examined. Cholera toxin increased the content of adenosine 3':5'-monophosphate (cAMP) in bone. The effect on cAMP was of slower onset and of longer duration as compared with parathyroid hormone (PTH) effect. PTH added to the tissue which had been stimulated by cholera toxin increased cAMP further but the effect was partially additive. In contrary to PTH which caused a clear calcium mobilization, cholera toxin by itself had no effect or rather inhibited the release of ^{45}Ca from the prelabeled bone. When the toxin (0.1-1 μg/ml) was combined with PTH (10 U/ml), calcium release stimulated by PTH was completely abolished.

It is well known that parathyroid hormone (PTH) and calcitonin given in vitro (1,2) or in vivo (3,4) induce an increased adenosine 3':5'-monophosphate (cAMP) content in the skeletal tissue. However, it has not been explained yet how cAMP is involved in the action of PTH to increase bone mineral mobilization and in the opposite action of calcitonin to decrease it. Though the administration of dibutyryl cAMP in vivo into thyroparathyroidectomized animals mimics the action of PTH inducing an increased plasma level of calcium (5,6), the results obtained in vitro on cultured skeletal tissue are complicated (7-10). Klein and Raisz (8) observed a biphasic action of dibutyryl cAMP on ^{45}Ca release from fetal bone and suggested a presence of two different pools of cAMP related to bone resorption and formation, respectively. Recently, Dietrich et al (10) raised a possibility that cAMP increased by PTH is related to the inhibition of osteoblastic activity and cAMP increased by calcitonin to the inhibition of osteoclastic activity.

In the present study, we examined whether cholera toxin could induce

ISSN 0006-291X

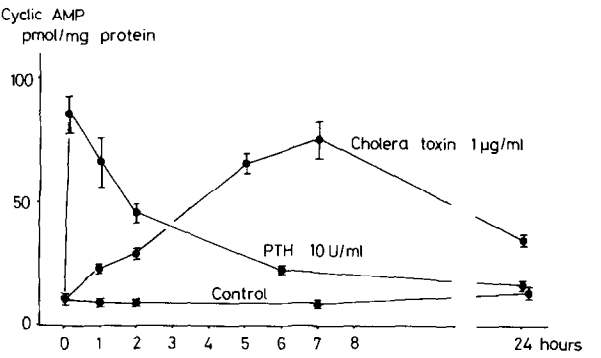

Fig. 1 Effects of cholera toxin and PTH on cAMP in bone. Rat fetal bone was cultured in control medium for 24 hours and then either saline (control), cholera toxin or PTH was added (0 time). A phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine 0.5 mM, was added into all incubations 30 min prior to the end of the reaction. Each mean ± SEM is of 4 cultures.

an increased cAMP in the skeletal tissue, and if so, how the change of cAMP affected bone mineral metabolism. Cholera toxin has been shown to stimulate adenylate cyclase in numbers of tissues and induce the accumulation of tissue cAMP (11-18).

METHODS AND MATERIALS

A pair of radius and ulna from each limb of fetus of 18-19th day of gestation was excised and cultured on stainless steel grid in 1 ml of Ham F 12 medium (Nissui Seiyaku Co., Tokyo) in an atmosphere of 5 % CO_2 and 95 % air at 37°C. The culture medium was modified to contain 1 mM calcium, 1 % bovine serum albumin, 5000 U/100 ml of penicillin and 5 mg/100 ml of streptomycin sulfate. After 24 hours of culture, the medium was changed to the same control culture medium or containing appropriate addition(s).

To examine the level of cAMP in bone, 0.5 mM of 3-isobutyl-1-methyl xanthine (Aldrich Chemical Co.) was added to the culture medium 30 min prior to the termination of reaction. The tissue was homogenized in cold 7.5 % trichloroacetic acid in glass homogenizer, and the extract was shaken for 3 times with water-saturated ethyl ether and neutralized further with 0.1 M Tris. The formed precipitate was removed by centrifugation and cAMP purified on Dowex 50-X8 (H^+ form) column was measured by Gilman's method as described elsewhere (19). Trichloroacetic acid precipitate was dissolved in 1 N NaOH and protein was determined by the method of Lowry et al (20) using bovine serum albumin as standard.

To measure the release of calcium, pregnant rat was injected intraperitoneally with 300 µCi of $^{45}CaCl_2$ (21.4 mCi/mg, New England Nuclear) on 17th day of pregnancy and was killed 24 hours thereafter. A pair of radius and ulna from each limb of fetus was incubated for 24 hours in control medium as described above. After this period of preculture, medium, control or containing an appro-

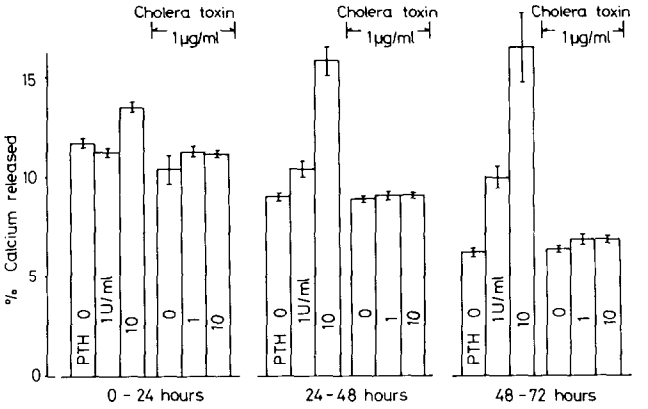

Fig. 2 Effects of cholera toxin and PTH on ^{45}Ca release from prelabeled bone. Prelabeled fetal bone was cultured for 24 hours in the control medium. Thereafter, the medium containing indicated addition was changed every 24 hours and ^{45}Ca in it was counted. The percentage release of total ^{45}Ca shown was determined as described in Methods. Each mean ± SEM is of 4 cultures.

priate addition, was changed every 24 hours for 3 days, and released ^{45}Ca during each 24 hours was counted in scintillation fluid containing 30 % Triton, 70 % toluene, 10 mg/100 ml 1,4-bis[2-(5-phenyl oxazolyl)] benzene and 0.4 g/100 ml 2,5-diphenyl oxazole. At the end of 3 days of this experimental culture, the tissue was homogenized in 0.3 ml of water in glass homogenizer and counted for radioactivity after dissolving in 0.5 ml of Protosol (New England Nuclear). The percentage release of total ^{45}Ca was determined by measured radioactivity in both bone and medium and was calculated as follows:

percent release = (medium ^{45}Ca/[medium ^{45}Ca + bone ^{45}Ca]) x 100.

PTH (trichloroacetic acid powder, 363 U/mg) and cholera toxin (lot No. CZ-2820) were purchased from Inolex Corp. and Schwarz/Mann, respectively.

RESULTS

Cholera toxin as well as PTH induced an increased cAMP content in bone (Fig. 1). The effect of cholera toxin was of slower onset and of longer duration as compared with that of PTH. The level of cAMP after the addition of PTH showed a peak at 5 min, leveled off to a half of the peak value already at 2 hours and was at the base line at 24 hours. On the otherhand, the levels of cAMP measured at 5 to 7 hours after the addition of cholera toxin were comparable to that found in the tissue exposed to PTH for 5 min, and still significantly higher than base line after 24 hours.

Table 1 shows the effect of varying concentration of cholera toxin as

Table 1

The cAMP content in the presence of varying concentration of cholera toxin and/or PTH

	Cyclic AMP pmol/mg protein			
	Cholera toxin μg/ml			
	0	0.05	0.5	1.0
None	17.6 ± 1.2	47.3 ± 2.8	102.6 ± 6.0	124.0 ± 7.3
PTH 10 U/ml	132.6 ± 12.9			177.8 ± 14.5

After 24 hours of preculture, media were changed to those containing cholera toxin at concentrations indicated and incubated for 5 hours thereafter. PTH was present for the last 5 min. 3-isobutyl-1-methyl xanthine (0.5 mM) was added to all incubations 30 min before the end of the reaction. Each mean ± SEM is of 4 cultures.

well as combined effect of the toxin and PTH on cAMP. Cholera toxin, at 5 hours after its addition, induced a dose dependent increase of cAMP within the range of dose examined. When PTH was allowed to work for 5 min on the tissue which had been stimulated by cholera toxin for 5 hours, cAMP was increased further but its effect was only partially additive.

Fig. 2 shows the release of calcium from prelabeled fetal bone. Culture medium containing indicated reagent(s) was changed every 24 hours and ^{45}Ca in it was counted. The stimulation of calcium release by 10 U/ml of PTH was evident within 24 hours and that by 1 U/ml of PTH was seen after 2nd day of culture. Cholera toxin, on the otherhand, was not only ineffective on calcium mobilization by itself but inhibitory to the effect of both high and low doses of PTH. The inhibitory effect was complete within 24 hours and continued until the third day of the culture. The concentration of cholera toxin used induced an increase of cAMP to the comparable level to that by 10 U/ml PTH (Fig. 1 and Table 1).

Table 2

Effect of the concentration of cholera toxin on PTH-induced calcium release

	% Calcium released					
	0-24 hours		24-48 hours		48-72 hours	
	PTH 10 U/ml					
	-	+	-	+	-	+
Cholera toxin						
0 μg/ml	11.4 ± 0.3	14.7 ± 0.6	9.4 ± 0.1	15.2 ± 0.6	8.1 ± 0.2	15.6 ± 0.5
0.05		12.6 ± 0.5		11.8 ± 0.6		12.2 ± 1.2
0.1		11.2 ± 0.8		9.4 ± 0.6		8.2 ± 0.4
0.5		11.5 ± 0.2		9.1 ± 0.1		7.6 ± 0.1
1.0	11.3 ± 0.2	10.8 ± 0.1	8.4 ± 0.3	8.4 ± 0.1	6.0 ± 0.3	7.1 ± 0.1

After 24 hours of preculture, media were changed to those containing indicated addition(s). Culture was continued for 3 days and released calcium during every 24 hours was determined. The percentage release of total ^{45}Ca shown was calculated as described in Methods. Each mean ± SEM is of 4 cultures.

The effect of varying dose of cholera toxin on PTH-induced calcium mobilization is summarized in Table 2. Antagonistic effect was clear by 0.05 μg/ml of cholera toxin already on the first day of addition and was complete by the presence of 0.1 μg/ml of the toxin. In this experiment, bone cultured in the presence of 1 μg/ml of the toxin added with or without PTH released less calcium than bone of control culture.

DISCUSSION

Cholera toxin induced an increased cAMP in rat fetal bone culture *in vitro*. In an accordance with a characteristic of the toxin response demonstrated in a variety of tissues (11-18), the effect was of slow onset and of long duration. As recent reports from a number of laboratories have indicated that most, if not all, of the biological effect of cholera toxin in the intestine as well as other organs are mediated via cAMP (11-18), we thought cholera toxin be useful to investigate the role of cAMP in bone mineral metabolism. It was found that cholera toxin powerfully inhibited the calcium mobilization stimulated by PTH. According to the popular hypothesis that cAMP is a second messenger of PTH action on bone resulting in bone mineral mobilization (21), the fact that cholera toxin antagonizes PTH effect is difficult to explain and is in marked contrast to the effects of the toxin observed in the other tissues examined. Cholera toxin has been shown to stimulate glycerol production in fat cells (12), glucose oxidation and colloid droplet formation in thyroid slices (15), glycogenolysis in liver tissue (22), steroidogenesis in adrenal cells (16) and testosterone production in rat testis (17), thus mimics the hormonal stimulation thought to be mediated via the adenylate cyclase-cAMP system.

As a combined administration of PTH and cholera toxin exhibited only a partial additivity on cAMP accumulation, it seems that the adenylate cyclase of PTH-sensitive cells is stimulated also by cholera toxin. It comes out, therefore, that the increase of cAMP in the cells including target cells of PTH by such a ubiquitous stimulator as cholera toxin is not enough to drive the cells

to mobilize bone mineral. Simply interpreted, the result is consistent with the notion that cAMP in bone is a second messenger of calcitonin action and its increase results in the suppression of bone mineral mobilization.

Recently, Luben et al (23) separating two metabolically distinct types of bone cell population showed that cells possessing adenylate cyclase sensitive to calcitonin were enriched in cells which expressed the metabolic characteristics of osteoclast and those with PTH-sensitive adenylate cyclase were enriched in cells characteristic of osteoblast. Their conclusion on the role of cAMP increased in each cell population by PTH or calcitonin seems to be consistent with the notion by Dietrich et al (10) that the PTH-stimulated increase in cAMP might result in decreased osteoblastic activity, whereas the calcitonin-stimulated increase might result in decreased osteoclastic activity. Such recent findings suggest that the stimulation by PTH of osteoclastic bone resorption is not mediated by cAMP. The finding in the present study that cholera toxin, probably through increased cAMP, inhibited PTH-induced bone resorption may support their conclusion (10,23) that cAMP suppresses the osteoclastic activity.

Although the cholera toxin-stimulated adenylate cyclase failed to mimic the effect of PTH and apparently reproduced the effect of calcitonin in bone, further study is needed to determine exactly the role of cAMP increased by each hormone. It seems most interesting to know whether bone mineral mobilization stimulated by PTH is actually through mechanism independent of cAMP or still mediated by cAMP working in concert with some signal(s) such as a change of intracellular calcium concentration (24).

REFERENCES

1. Chase,L.R., and Aurbach,G.D. (1970) J. Biol. Chem. 245 1520-1526.
2. Murad,F., Brewer,H.B.,Jr., and Vaughan,M. (1970) Proc. Natl. Acad. Sci. US. 65 446-453.
3. Nagata,N., Sasaki,M., Kimura,N., and Nakane,K. (1975) Endocrinology 96 725-731.
4. Nagata,N., Sasaki,M., Kimura,N., and Nakane,K. (1975) Endocrinology 97 527-535.

5. Wells,H., and Lloyd,W. (1969) Endocrinology 84 861-867.
6. Rasmussen,H., Pechet,M., and Fast,D. (1968) J. Clin. Invest. 47 1843-1850.
7. Vaes,G. (1968) Nature 219 939-940.
8. Klein,D.C., and Raisz,L.G. (1971) Endocrinology 89 818-826.
9. Herrmann-Erlee,M.P.M., and Meer,J.M. (1974) Endocrinology 94 424-434.
10. Dietrich,J.W., Canals,E.M., Maina,D.M., and Raisz,L.G. (1976) Endocrinology 98 943-949.
11. Hynie,S., and Sharp,G.W.G. (1972) in Advances in Cyclic Nucleotide Research, Vol 1, (Greengard,P., Robison,G.A., and Paolelli,R., eds) pp.163-174, Raven Press, New York.
12. Vaughan,M., Pierce,N.F., and Greenough,W.B.,III. (1970) Nature 226 658-659.
13. Gorman,R.E., and Bitensky,M.W. (1972) Nature 235 439-440.
14. Strom, T.B., Deisseroth,A., Morganroth,J., Carpenter,C.B., and Merrill,J.B. (1972) Proc. Natl. Acad. Sci. US. 69 2995-2999.
15. Mashiter,K., Mashiter,G.D., Hauger,R.L., and Field,J.B. (1973) Endocrinology 92 541-549.
16. Haksar,A., Maudsley,D.V., and Peron,F.G. (1975) Biochim. Biophys. Acta 381 308-323.
17. Sato,K., Miyachi,Y., Ohsawa,N., and Kosaka,K. (1975) Biochem. Biophys. Res. Comm. 62 696-703.
18. Kurokawa,K., Friedler,R.M., and Massry,S.G. (1975) Kidney International 7 137-144.
19. Kakuta,S., Suda,T., Sasaki,S., Kimura,N., and Nagata,N. (1975) Endocrinology 97 1288-1293.
20. Lowry,O.H., Rosebrough,N.J., Farr,A.L., and Randall,R.J. (1951) J. Biol. Chem. 193 265-275.
21. Aurbach,G.D., Keutmann,H.T., Niall,H.D., Tregear,G.W., O'Riordan,J.L.H., Marcus,R., Marx,S.J., and Potts,J.T.,Jr. (1972) Recent Progress in Hormone Research 28 353-398.
22. Zieve,P.D., Pierce,N.F., and Greenough,W.B. (1970) Clin. Res. 18 690.
23. Luben,R.A., Wong,G.L., and Cohn,D.V. (1976) Endocrinology 99 526-534.
24. Rasmussen,H. (1971) Am. J. Med. 50 567-588.