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THE DIRECT INVOLVEMENT OF cAMP-DEPENDENT PROTEIN KINASE IN THE REGULATION OF COLLAGEN SYNTHESIS BY PARATHYROID HORMONE (PTH) AND PTH-RELATED PEPTIDE IN OSTEOBLAST-LIKE OSTEOSARCOMA CELLS (UMR-106)

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The present study was performed to characterize the direct involvement of cAMP-dependent protein kinase (PKA) in the regulation of collagen synthesis by parathyroid hormone (PTH) and PTH-related peptide (PTHrP) in osteoblastic osteosarcoma cells, UMR-106. Sp-cAMPS (10^{-4}M), a direct activator of PKA, as well as dibutyryl cAMP (dbcAMP, 10^{-4}M) significantly inhibited collagen synthesis. Human (h) PTH-(1-34) (10^{-7}M) and hPTHrP (10^{-7} M) inhibited collagen synthesis to the same degree. Although Rp-cAMPS, which acted directly as an antagonist in the activation of PKA, did not affect collagen synthesis by itself, it significantly antagonized dbcAMP- and Sp-cAMPS-induced inhibition of collagen synthesis. Moreover, Rp-cAMPS antagonized PTH- and PTHrP-induced inhibition of collagen synthesis to the same degree. The present study first indicated that the activation of PKA was directly linked to the regulation of collagen synthesis by PTH in osteoblast and that PTHrP had the same effect on collagen synthesis presumably through the same mechanism as PTH.

In the state of excess of parathyroid hormone (PTH), such as primary hyperparathyroidism, loss of bone mass is remarkable. Bone mass is regulated by total amount of cellular component, such as osteoblast, osteoclast, and bone matrix. Bone matrix consist of inorganic component and organic component which mainly contains type 1 collagen. Therefore, the regulation of collagen synthesis in osteoblasts is important to maintain bone mass. There have been several lines of evidence that collagen synthesis by osteoblasts is mediated by multihormonal regulation and PTH inhibits collagen synthesis in bone organ cultures and osteoblastic cell lines (1-5). Previous evidence suggested that cAMP might be involved in the regulation of

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Abbreviations: cAMPS, adenosine 3',5'-monophosphorothioate; EDTA, ethylene diaminetetraacetic acid.

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collagen synthesis by PTH (1,3), but no reports are available now about the direct involvement of cAMP-dependent protein kinase (PKA) in the regulation of collagen synthesis by PTH. Recently, the diastereoisomers of the phosphorothioate analogue of cAMP, Sp-cAMPS and Rp-cAMPS which directly stimulated and inhibited PKA, respectively, were developed and employed to examine hormone-stimulated cellular responses and to distinguish cAMP-dependent from cAMP-independent events (6,7). We first employed these compounds on bone cells and demonstrated the direct involvement of PKA in the regulation of osteoblast proliferation by PTH and PTH-related peptide (PTHrP) in osteoblastic osteosarcoma cells, UMR-106 (8,9). Present study was performed to clarify whether or not the activation of PKA would be directly linked to the regulation of collagen synthesis by PTH in UMR-106. Experiments were also done, using PTHrP, a causative peptide associated with humoral hypercalcemia of malignancy, which had similar amino acid sequencing as PTH at amino-terminus (10,11) and the effect of PTHrP was compared to that of PTH.

Materials and Methods

Materials

UMR-106 cells were the generous gift from Dr. T. J. Martin (Melbourne, Australia). Human (h)-PTH-(1-34) and hPTHrP-(1-34) were obtained from Peptide Institute Inc. (Osaka, Japan). Sp-cAMPS and Rp-cAMPS from Biolog Life Science Institute (Bremen, Germany), N^6, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP), phorbol 12-myristate 13-acetate (PMA) and ionomycin from Sigma Co. (St. Louis, MO), A23187 from Hoechst Japan Co. (Tokyo, Japan), L-[2,3,(n)-^{3}H] proline from Amersham Japan (Tokyo, Japan), bacterial collagenase from Advance Biofactures Co. (Lynbrook, NY). All other chemicals were of analytical grade.

Cell culture

UMR-106 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum in a 5% CO_2-95% air atmosphere at 37^{0}C, as previously described (12). Cells were weekly passed using 0.05% trypsin-0.02% EDTA solution. For experiments, cells were cultured in 6-well plates.

Collagen assay

Twenty-four hrs after treatment with indicated concentration of substances, the cells were pulsed with [^{3}H]proline (2μCi/ml) under co-existence of 0.5mM β-aminopropionitrile and 0.5mM ascorbic acid. Three hrs later, the incubation was terminated by removal of the medium and the addition of 10% trichloroacetic acid (TCA). The cells were scraped by rubber policeman and transferred to tubes. Protein was extracted by the addition of 10% TCA and acetone. Desiccated protein was digested by collagenase at 37^{0}C for 90 min. Supernatant was took out and scintillation cocktail was added. Samples were counted in a liquid scintillation counter. Data were expressed as the mean ± SEM. Statistical analysis was performed, using student's t test or Duncan's multiple range test

Results and Discussion

First, the effect of PKA activation on collagen synthesis was examined in UMR-106 cells. As shown in figure 1, Sp-cAMPS(10^{-4}M), a direct activator of PKA as well as *dbcAMP* (10^{-4}M) significantly inhibited [^{3}H] proline incorporation into collagenase-digestible protein (CDP) in these cells, indicating that Sp-cAMPS mimicked

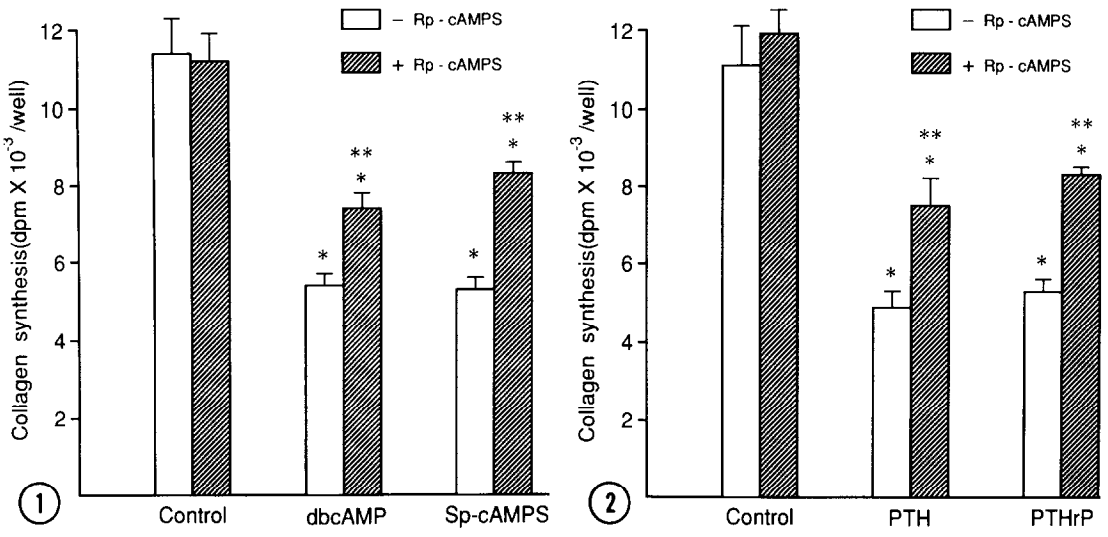

FIG. 1. Effect of dbcAMP and Sp-cAMPS on collagen synthesis in the presence or absence of Rp-cAMPS. Collagen synthesis was measured as described in Materials and Methods. Cells were treated with dbcAMP (10^{-4}M) or Sp-cAMPS (10^{-4}M) 30 min after the addition of Rp-cAMPS (10^{-4}M). Each bar represents the mean ± SEM of six determinations. * P <0.01, compared to control. ** P<0.01, compared to each Rp-cAMPS-untreated group.

FIG. 2. Effect of PTH and PTHrP on collagen synthesis in the presence or absence of Rp-cAMPS. Cells were treated with hPTH-(1-34) (10^{-7}M) or hPTHrP-(1-34) (10^{-7}M) 30 min after the addition of Rp-cAMPS (10^{-4}M). Each bar represents the mean ± SEM of six determinations. * P<0.01, compared to control. ** P<0.01, compared to each Rp-cAMPS-untreated cells.

cAMP-induced effect on collagen synthesis. On the other hand, Rp-cAMPS(10^{-4}M), which acted directly as an antagonist in the activation of PKA, did not affect its incorporation into CDP by itself, but it significantly antagonized the inhibitory effect on its incorporation into CDP induced by dbcAMP and Sp-cAMPS (figure 1), indicating that the activation of PKA was directly coupled to the inhibition of collagen synthesis. Next, effects of PTH and PTHrP on collagen synthesis was examined. There has been previous evidence that PTH and PTHrP caused the increase in cAMP accumulation of UMR-106 cells to the same degree (13) and our preliminary study reconfirmed this phenomenon (data not shown). As shown in figure 2, hPTH-(1-34) (10^{-7}M) and hPTHrP-(1-34) (10^{-7}M) significantly inhibited its incorporation into CDP to the same degree. Present data was incompatible with recent evidence from organ culture that PTHrP was less potent than PTH in inhibiting collagen synthesis (14). This discrepancy might be ascribed to the differences of cells used and experimental protocol. Rp-cAMPS (10^{-4}M) antagonized PTH- and PTHrP-induced inhibition of its incorporation into CDP to the same degree (figure 2). These results first indicated that the activation of PKA was directly linked to the regulation of collagen synthesis by PTH in osteoblasts and that PTHrP inhibited collagen synthesis presumably through the same mechanism as PTH. In the present study, however, Rp-cAMPS did not

completely antagonize the cAMP analogues-, PTH- and PTHrP-induced inhibitory effect on collagen synthesis in these cells. As discussed in our recent reports (8,9), three possibilities must be considered. First, Rp-cAMPS even at 10^{-4}M was not potent enough to completely antagonize the activation of PKA. Second, Rp-cAMPS has been reported to inhibit phosphodiesterase activity in cultured Leydig tumor cells (15). Therefore, it is possible that a partial inhibition of phosphodiesterase activity in turn might allow endogenous cAMP to accumulate, resulting in partial compensation of PKA inhibition. Third, it might be presumed that PKA is not the only signal transduction system involved in the regulation of collagen synthesis by PTH and PTHrP in osteoblasts. Indeed, it has been reported that PTH acts on polyphosphoinositide metabolism, another signal transduction system, resulting in the elevation of cytosolic calcium and the activation of PKC (Ca/PKC) in UMR-106 cells (16,17). PTHrP also has been reported to elevate cytosolic calcium in these cells (18). To clarify whether or not this transduction system also played a role in the regulation of collagen synthesis in these cells, we employed PMA which activated PKC and calcium ionophores (A23187 and ionomycin) which increased the cytosolic calcium. As shown in figure 3, PMA (10^{-6}M) did not affect [^{3}H]proline incorporation into CDP, but both A23187 (10^{-6}M) and ionomycin (10^{-6}M) significantly inhibited its incorporation into CDP. These results indicated that the increase in cytosolic calcium might be involved in the regulation of collagen synthesis by PTH and PTHrP in these cells, although we could not completely rule out the possibility that these calcium ionophores-induced effect might be mediated by some other mechanism that the increase in cytosolic calcium. Present study did not clarify how these dual signal transduction systems (PKA and Ca/PKC) cooperated to manifest the regulation of collagen synthesis by PTH and PTHrP. Further studies are necessary to clarify it and these studies are in progress in our

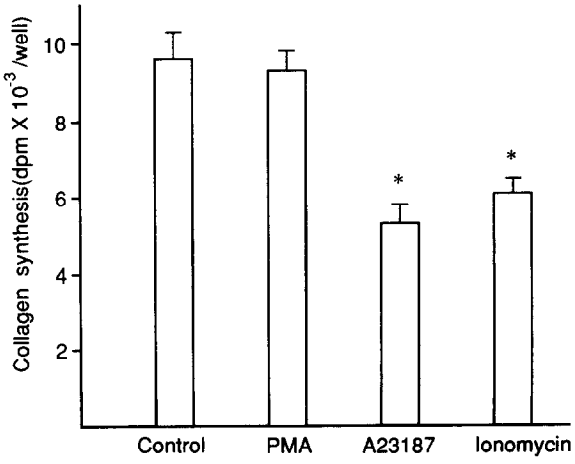

FIG. 3. Effect of PMA, A23187 and ionomycin on collagen synthesis. Cells were treated with PMA (10^{-6}M), A23187 (10^{-6}M) or ionomycin (10^{-6}M) for 24 hrs. Each bar represents the mean ± SEM of six determinations. * $P<0.01$, compared to control.

laboratory. In conclusion, the activation of PKA was directly linked to the regulation of collagen synthesis by PTH in osteoblasts and PTHrP had the same inhibitory effect on collagen synthesis, presumably through the same mechanism as PTH.

References

1. Dietrich, J.W., Canalis, E.M., Maina, D.M., and Raisz, L.G. (1976) Endocrinology 98, 943-949.
2. Kream, B.E., Rowe, D., Smith, M.D., Maher, V., and Majeska, R. (1986) Endocrinology 119, 1922-1928.
3. Rosen, D.M., and Luben, R.A. (1983) Endocrinology 112, 992-999.
4. Pun, K.K. (1989) J. Biochem. 106, 1090-1093.
5. Partridge, N.C., Dickson, C.A., Kopp, K., Teitelbaum, S.L., Crouch, E.C., and Kahn, A.J. (1989) Molecular Endocrinology 3, 232-239.
6. Rothermel, J.D., Jastorff, B., and Botelho, L.H.P. (1984) J. Biol. Chem. 259, 8151-8155.
7. Erneux, C., Van Sande, J., Jastorff, B., and Dumont, J.E. (1986) Biochem. J. 234, 193-197.
8. Kano, J., Sugimoto, T., Fukase, M., and Fujita, T. (1991) Biochem. Biophys. Res. Commun. 177, 365-369.
9. Kano, J., Sugimoto, T., Fukase, M., and Fujita, T. (1991) Biochem. Biophys. Res. Commun. 179, 97-101.
10. Moseley, J.M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R.E.H., Kemp, B.E., Suva, L.J., Rodda, C.P., Ebeling, P.R., Hudoson, P.J., Zajac, J.D., and Martin, T.J. (1987) Proc. Natl. Acad. Sci. USA 84,5048-5052.
11. Suva, L.J., Winslow, G.A., Wettenhall, R.E.H., Hammonds, R.G., Moseley, J.M., Diefenbach-Jagger, H., Rodda, C.P., Kemp, B.E., Rodriguez, H., Chen, E.Y., Hudson, P.J., Martin, T.J., and Wood, W.I. (1987) Science 237,893-896.
12. Sugimoto, T., Nakada, M., Fukase, M., Imai, Y., Kinoshita, Y., and Fujita, T. (1986) Calcif. Tissue Int. 39, 171-174.
13. Kemp, B.E., Moseley, J.M., Rodda, C.P., Ebeling, P.R., Wettenhall, R.E.H., Stapleton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V.P., Simmons, H.A., Raisz, L.G., Martin, T.J. (1987) Science 238,1568-1570.
14. Klein-Nulend, J., Fall, P.M., and Raisz, L.G. (1990) Endocrinology 126, 223-227.
15. Pereira, M.E., Segaloff, D.L., Ascoli, M., and Eckstein, F. (1987) J. Biol. Chem. 262, 6093-6100.
16. Civitelli, R., Reid, I.R., Westbrook, S., Avioli, L.V., and Hruska, K.A. (1988) Am. J. Physiol. 255, E660-E667.
17. Reid, I.R., Civitelli, R., Halstead, L.R., Avioli, L.V., and Hruska, K.A. (1987) Am. J. Physiol. 252, E45-E51.
18. Civitelli, R., Martin, T.J., Faust, A., Gunsten, S.L., Hruska, K.A., and Avioli, L.V. (1989) Endocrinology 125,1204-1210.