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The Effect of Pulsed Electromagnetic Fields on the Calcium Metabolism in Cultured Rabbit Chondrocytes

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Summary. **We have previously demonstrated that pulsed electromagnetic fields (PEMF) affect the functional differentiation of rabbit growth plate chondrocytes in culture by enhancing the response of the parathyroid hormone (PTH). In this study, we examined whether the PEMF affect the proliferation of cultured chondrocytes as well as how their effect on the PTH response of the chondrocytes is related to the calcium metabolism. We cultured growth plate chondrocytes isolated from the ribs of young rabbits and treated them with Verapamil, a calcium blocker, under exposure to PEMF (pulsating recurrent burst, an average of 2 gauss). No significant effect on [^{3}H]-thymidine incorporation into DNA synthesis in either the confluent or logarithmic phase was observed under exposure to PEMF. Verapamil: 1) inhibited DNA synthesis dose-dependently, whereas it did not change the level of glycosaminoglycan (GAG) synthesis; and 2) did not alter the level of PTH-dependent GAG synthesis in the presence or absence of PEMF. These findings indicated that PEMF stimuli resulted in the functional differentiation of growth plate chondrocytes by enhancing GAG synthesis in response to PTH, and that this effect was not affected by the change in the concentration of calcium ions.**

Key words—pulsed electromagnetic fields, chondrocyte, Verapamil, PTH.

INTRODUCTION

There are many reports on the use of pulsed electromagnetic fields (PEMF) for inducing osteogenesis or stimulating the growth of skeletal tissues or repair in fractures. However, the effect and mechanism of PEMF at the cellular level are unknown.

We have demonstrated that PEMF affects the differentiated phenotype of rabbit costal chondrocytes in culture by enhancing the response of PTH (2.5IU/ml). In a previous study we showed that: 1) intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation in response to PTH was elevated markedly by applying pulsed electromagnetic fields in cultured rabbit chondrocytes; 2) induction of ornithine decarboxylase (ODC) in response to PTH was enhanced; and 3) GAG synthesis in response to PTH was also significantly enhanced in the presence of PEMF.[1] These results suggested that PEMF stimulated functional differentiation or the expression of a differentiated phenotype of chondrocytes by enhancing the hormonal action of PTH. Also, cAMP appeared to play an important role as one of the mediators of the actions of PEMF. On the other hand, calcium (Ca^{2+}) concentration has been postulated as a modulator of mitotic activity.[2-4]

In order to examine whether the effect of PEMF on the enhancement of PTH action is mediated through calcium ion, we investigated the effect of PEMF and Verapamil, a calcium blocker, on DNA and GAG synthesis treated with PTH in cultured rabbit chondrocytes.

MATERIALS AND METHODS

Materials

Bovine PTH-active fragment (synthetic 1-34 : 6000 IU/mg) was obtained from Beckman Instruments Inc. (USA). Eagle's minimum essential medium (MEM) and fetal calf serum (FCS) were acquired

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from Nissui Pharmaceutical Co., (Tokyo, Japan) and GIBCO, (USA), respectively. [^{35}S]-sulfic acid (carrier free) came from the Japan Atomic Energy Research Institute, Japan, and [^{3}H]-thymidine from New England Nuclear, USA. Materials used in this study were commercial products of the highest grade available (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Cell culture and PEMF stimuli

Chondrocytes were isolated from growth plate cartilage of the ribs of young male New Zealand rabbits, weighing 300-600 g, as described previously.[1] The isolated cells were plated at a density of 8×10^4 cells per 35-mm diameter dish and grown in 2 ml of MEM supplemented with 10% FCS (previously heat-inactivated at 56°C) at 37°C under 5%CO_2 in the open air.

PEMF was generated in Helmholz coils 35-mm diameter, with pulsating recurrent currents of a 5 ms burst of 210 msec and 25 msec opposite polarity repeated at 15.4 Hz. Each 35-mm culture dish was placed between each Helmholz coil placed vertically in relation to the culture dish. The measured strength of PEMF was an average of 2.4 gauss in the culture dish at the center of the helmholtz coils.

Determination of DNA synthesis

Cultured cells were labeled at 37°C with 1.0 uCi/m [$^{6-3}$H] thymidine for 2 h. The cells were washed three times with cold phosphate buffered saline (PBS) and precipitated with 5% trichloroacetic acid (TCA), then solubilized with 0.3 N NaOH and neutralized with 6 N HCl. The radioactivity was measured to evaluate DNA synthesis using a liquid scintillater (Aloka LSC-3050).

Determination of GAG synthesis

After each experiment, the cultured cells were rinsed with Gey's solution containing 10% (v/v) Hanks solution, and then labeled with the same solution containing [^{35}S] sulfate (2 uCi/ml) for 4 h. The cell layers were dissolved with ice-cold 0.1 M NaOH and collected with a rubber policeman. Glycosaminoglycans in the labeling medium and in the cell layer were precipitated with cetylpyridinium chloride, separately. The radioactivity in the precipitates was measured with a liquid scintillater (Aloka LSC-3050). A portion of the neutralized-cell lysate was used for protein assay by the Lowry method.[5]

Effect of PEMF on DNA synthesis

When the chondrocytes were cultured at an initial density of 1×10^5 cells in the 35 mm dishes to reach a confluent phase or logarithmic phase, PEMF was applied to the culture dish, followed by incubation for 48 h. The rate of DNA synthesis of cultured chondrocytes was then measured by labeling with 1.0 uCi/ml of [^{3}H] thymidine for the last 2 h.

The effect of PEMF and Verapamil on the PTH-induced GAG synthesis

When chondrocytes reached a subconfluent phase, the cells were incubated under PEMF exposure for 48 h. Glycosaminoglycan synthesis was measured to evaluate the effect of PEMF on the differentiation of chondrocytes. Verapamil (10^{-7}-10^{-3} M) was added in the medium, and then [^{3}H] thymidine incorporation and [^{35}S] sulfate incorporation were measured to evaluate DNA and GAG synthesis, respectively.

Statistical analysis

Results were expressed as mean$\pm$1SD and compared using the ANOVA test. A significance was determined at $p<0.05$.

RESULTS

Effect of PEMF on DNA synthesis

In order to examine the effect of PEMF on cell proliferation, cells were grown for 48 h under constant exposure to PEMF. Fig. 1 shows that no difference in thymidine incorporation was observed in either the logarithmic growth states or the confluent states.

Effect of Verapamil on GAG and DNA synthesis

We examined the effect of calcium ion (Ca^{2+}) on GAG and DNA synthesis in rabbit costal chondrocytes using Verapamil, a calcium blocker. Fig. 2 shows the effects of Verapamil on GAG and DNA synthesis in the absence of PEMF. No significant change in the GAG synthesis of cultured chondrocytes treated with Verapamil (10^{-7}-10^{-3} M) was observed, whereas DNA synthesis was inhibited with the addition of Verapamil (10^{-4}-10^{-3} M).

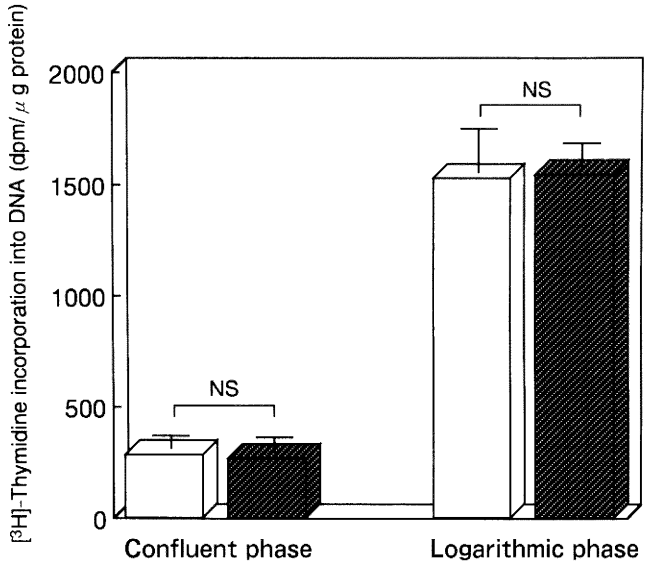

Fig. 1. The effect of a 48-hour exposure to PEMF on the [^{3}H]-thymidine incorporation into DNA in cultured chondrocytes. When the chondrocytes were cultured at an initial density of 1×10^5 cells in 35 mm dishes to reach a logarithmic or confluent state, they were continuously exposed to PEMF for 48 h. The open column shows the control group with no exposure to PEMF, and the hatched column shows the treated group with exposure to PEMF for 48 h. NS, difference is not statistically significant. (n=3)

Effect of Verapamil on PTH induced GAG synthesis in the presence or absence of PEMF

As previously reported,[1] PEMF increased GAG synthesis by chondrocytes treated with PTH. Consistent with previous findings,[1] GAG synthesis increased in response to PTH, and this GAG synthesis in response to PTH with exposure of PEMF was enhanced. We examined the effect of PTH and Verapamil (10^{-4} M) on GAG synthesis in the presence or absence of PEMF. As shown in Fig. 3, GAG synthesis increased significantly with PTH treatment compared with the control (PBS treated), and further increased in the presence of PEMF. However, PTH-induced GAG synthesis was not affected by Verapamil treatment during exposure to PEMF for 48 h.

DISCUSSION

Electric fields have been used to stimulate the fracture repair and growth of skeletal tissues.[2,6] The growth plate chondrocytes play an important role in the growth by humoral and mechanical stimuli, including electrical stimuli. We have previously reported that PEMF exposure enhanced cAMP accumulation, ornithine decarboxylase activity and GAG syn-

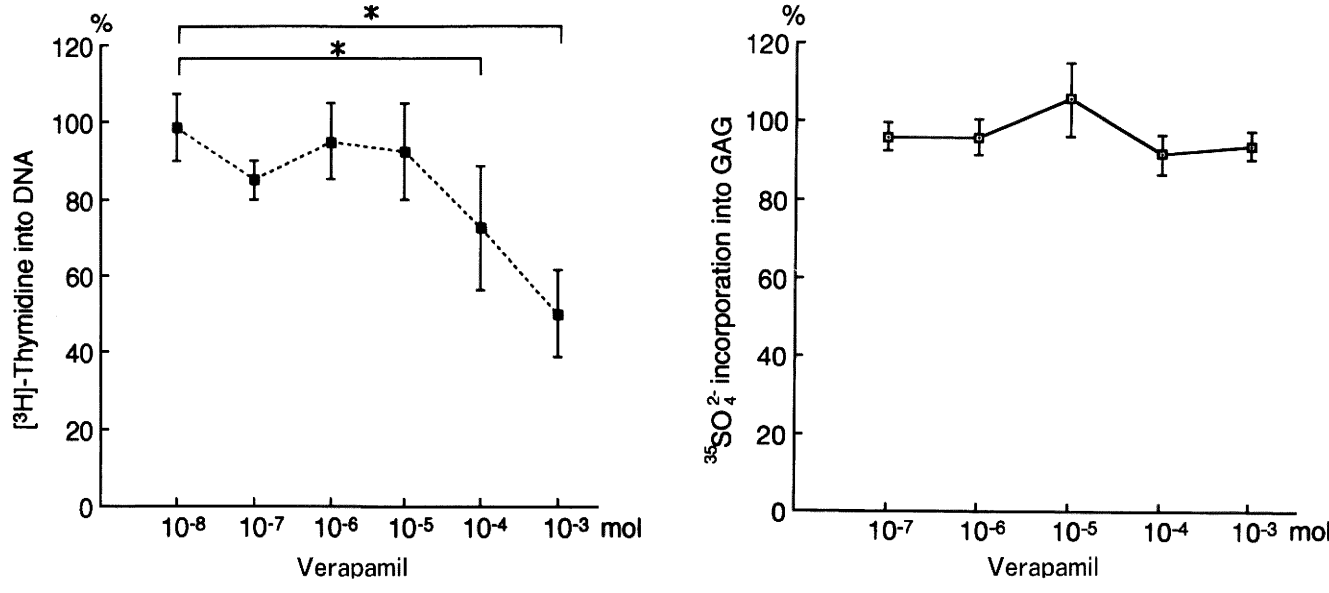

Fig. 2. DNA synthesis. (left) GAG synthesis. (right) The effect of Verapamil on the GAG synthesis and [^{3}H]-thymidine incorporation into DNA in cultured chondrocytes. When the chondrocytes were cultured at an initial density of 1×10^5 cells in 35 mm dishes to reach subconfluency, they were treated with Verapamil. Each value is the average $\pm$1SD in the three studies. *, a significance is reported at $p<0.05$.

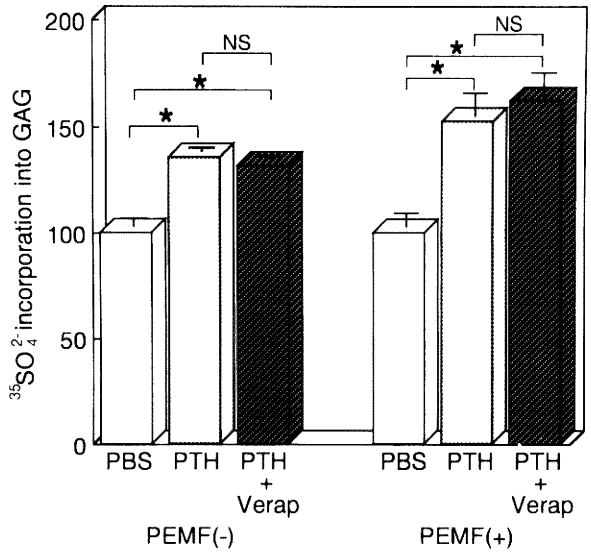

Fig. 3. The effect of a 48-hour exposure to PEMF on GAG synthesis in cultured chondrocytes. When the chondrocytes were cultured at an initial density of 1×10^5 cells in 35 mm dishes to reach subconfluency, they were continuously exposed to PEMF for 48 h. After treatment with PTH (2.5 IU/ml) alone, or Verapamil (10^{-4} M) and PTH (2.5 IU/ml), they were exposed to PEMF for 24 h. Each value is the average ±1SD (percent of control) in the three studies. The open column shows the control (treatment with PBS: phosphate buffered saline) group with no exposure to PEMF, the hatched column shows the treated group with PTH alone with exposure to PEMF for 48 h, and the dotted column shows PTH and Verapamil treatment with PEMF exposure. NS, the difference is not statistically significant. *, a significance is reported at $p<0.05$. ($n=3$)

thesis, and that these inductions in response to PTH are good markers of the differentiated phenotype of chondrocyte, suggesting that PEMF stimulates the functional differentiation of growth plate chondrocytes in culture.[1] However, the effect of PEMF on their proliferation and the effect of PEMF on calcium flux are unknown.

In this study, we found that: 1) the pulsed electromagnetic fields have no influence on DNA synthesis in logarithmic growth states or confluent states; 2) Verapamil, a calcium blocker, inhibited [^{3}H]-thymidine incorporation into DNA synthesis; and 3) Verapamil did not alter the level of PTH-induced GAG synthesis in the presence of PEMF. These findings show that the exposure of cultured rabbit chondrocytes to PEMF did not induce mitotic activity. Furthermore, PTH-dependent GAG synthesis in the presence of PEMF was not affected by the calcium blocker, Verapamil. In this study, we did not measure intracellular calcium levels; however, the results shown here indicate that the change in the calcium flux is not involved in PTH dependent GAG synthesis by PEMF. CAMP appears to be a secondary intracellular mediator in applying PEMF to cultured chondrocytes, and at least 48 hours' exposure of chondrocytes to PEMF was necessary for the enhancement of PTH responsiveness.[1] These findings suggest that PTH affect primarily adenylate cyclase and the PTH receptor by the change in PTH-sensitization at the cell membrane level, though the exact mechanism remains to be determined. Using Verapamil, Rodan et al. found that: 1) electrical fields induce membrane depolarization; 2) intracellular Ca^{2+} rises; 3) cAMP declines; and 4) DNA synthesis is initiated shortly after exposure.[3] We do not know whether the discrepancy between this report and our results is due to a difference in the cells used or a difference in electrical stimulation.[2,3,7]

Luben et al. reported the inhibition of PTH-stimulated cAMP following PEMF exposure in MMB-1 osteoblast-like cells "in vitro".[8] Brighton et al. reported that a small capacitively coupled electrical stimulation significantly decreases bone cell production of cAMP in response to PTH.[2,9,10] Furthermore, this decrease in cAMP response to PTH was greater after a 30-min delay between the cessation of the electrical field and application of PTH.[9,10,11] These results indicated that PEMF has varying effects on cAMP accumulation in response to PTH. Our studies and the above reports suggest that electrically induced osteogenesis may be mediated by changing the sensitization to hormonal action associated with the interaction of cells and electrical stimulation.

The present findings showed that PTH-dependent GAG synthesis by PEMF was not affected by the change in calcium fluxes.

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REFERENCES

1) Hiraki Y, Endo N, Takigawa M, Asada A, Takahashi H, Suzuki F: Enhanced responsiveness to parathyroid hormone and induction of functional differentiation of cultured rabbit costal chondrocytes by a pulsed electromagnetic field. *Biochemi Biophys Acta* **931:** 94-100, 1987.
2) Brighton CT, McCluskey WP: Cellular response and mechanisms of action of electrically induced osteogenesis. In: Peck WA (ed) Bone and Mineral Research 4. Elsevier, Amsterdam 1986, p. 213-254.
3) Rodan GA, Bourret LA, Norton LA: DNA synthesis in cartilage cells is stimulated by oscillating electric fields. *Science* **199:** 690-692, 1978.
4) McLeod KL, Donahue HL, Levin PE, Fontain MA, Rubin CT: Electric fields modulate bone cell function in a density-dependent manner. *J Bone Miner Res* **8:** 977-984, 1993.
5) Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265-275, 1951.
6) Takano-Yamamoto T, Kawakami M, Sakuda M: Effect of a pulsing electromagnetic field on demineralized bone-matrix-induced bone formation in a body defect in the premaxilla of rats. *J Dent Res* **71:** 1920-1925, 1992.
7) Yamada S, Guenther HL, Fleisch H: The effect of pulsed electromagnetic fields on bone cell metabolism and calvaria resorption in vitro, and on calcium metabolism in the live rat. *Int Orthop* **9:** 129-134, 1985.
8) Luben RA, Caine CD, Chen M C-Y, Rosen DM, Adey WR: Effects of electromagnetic stimuli on bone and bone cells in vitro: Inhibition of responses to parathyroid hormone by low-energy low-frequency fields. *Proc Natl Acad Sci USA* **79:** 4180-4184, 1982.
9) Brighton CT, Jensen L, Pollack SR, Tolin BS, Clark CC: Proliferative and synthetic response of bovine growth plate chondrocytes to various capacitively coupled electrical fields. *J Orthop Res* **7:** 759-765, 1989.
10) Brighton CT, McCluskey WP: Response of cultured bone cells to a capacitively coupled electric field: Inhibition of cAMP response to parathyroid hormone. *J Orthop Res* **6:** 567-571, 1988.
11) Pienkowski D, Pollack SR, Brighton CT, Griffith NJ: Low-power electromagnetic stimulation of osteomized rabbit fibulae. A randomized, blinded study. *J Bone Joint Surg Am* **76:** 489-501, 1994.