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Stimulation of cell proliferation in skeletal tissues of the rat by defined parathyroid hormone fragments

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We have found, in previous studies *in vitro* using skeletal derived cell cultures, that mid-region fragments of human parathyroid hormone (hPTH) stimulate [^{3}H]thymidine incorporation into DNA and increase the specific activity of the brain-type isoenzyme of creatine kinase (CK). These changes occurred without an increase in cyclic AMP formation which is linked to bone resorption. In this study, we found that the mid-region fragment hPTH-(28–48) stimulated CK activity in diaphysis, epiphysis and kidney in a time- and dose-dependent manner, parallel to the effects of the whole molecule bovine (b)PTH-(1–84) and the fully active fragment hPTH-(1–34). The increase caused by hPTH-(28–48) at a dose of 1.25 μg/rat was not less than the 2-fold increase caused by a roughly equimolar concentration of bPTH-(1–84). A significant increase was reached at 1 h after intraperitoneal injection in all cases. All three sequences of PTH caused an increase in [^{3}H]thymidine incorporation into DNA in diaphysis and epiphysis, but not in kidney, 24 h after injection. A fragment further towards the *C*-terminal, hPTH-(34–47), was inactive compared with an equimolar concentration of the fragment hPTH-(25–39), which stimulated both CK activity and DNA synthesis. These results *in vivo* are in line with previous findings *in vitro*; they provide further support for the suggestion that mid-region fragments of the PTH molecule could be used to induce bone formation without incurring the deleterious effect of bone resorption.

INTRODUCTION

Parathyroid hormone (PTH) has been shown to stimulate both formation [1] and resorption [2] of bone; the result of prolonged PTH treatment *in vivo* is an increase in bone turnover [3,4]. However, specific binding of PTH can be demonstrated only in osteoblasts [5] and osteoclast precursor cells [6], but not in mature osteoclasts. PTH has been shown to have a variety of actions *in vitro*, such as enhancement of cyclic AMP production [7] and cyclic AMP-dependent protein kinase activity [8], induction of changes in cell shape [9], stimulation of plasminogen activator [10], inhibition of collagen synthesis [11], suppression of alkaline phosphatase activity [12], and stimulation of cell proliferation [13,14] and enzymes associated with cell proliferation [15] such as ornithine decarboxylase [16,17] and the brain-type isoenzyme of creatine kinase (CK) [13,14].

PTH also causes increased proliferation of thymus, bone marrow and liver *in vivo* [18], increases which are mediated by changes in Ca^{2+} and/or cyclic AMP concentrations, in a variety of cell culture systems [8,17]. As well as increasing adenylate cyclase activity [19], PTH increases the proliferation [13,14] of cultured bone cells, osteosarcoma cells and chondroprogenitor cells [20]. PTH was also shown to modulate phospholipid metabolism [21]. It promotes bone formation at low doses *in vivo* [22–24], in addition to its effects on resorption; the net effect of the hormone on bone mass must, therefore, be determined by the balance between these two processes [25,26]. Using histological techniques, it was found that there was an increase in the bone apposition rate, accompanied by an increase in the formation surface, without any increase in the resorption surface, after daily injection of PTH-(1–84) into rats [27]. Moreover, bovine (b)PTH-(1–34) was shown to have anabolic activity [28] which did not depend on early stimulation of resorption [29]. Indeed, it has been shown that PTH, together with vitamin D_3, can be used at low doses for treatment of osteoporosis [30]. Moreover, increased bone formation in hyperparathyroid states in man [31] and animals [23,24] has also been demonstrated. Recently we have shown that the central portion of the PTH molecule increases cell proliferation in chondrocytes [32] and in osteoblasts [33] without affecting cyclic AMP production. Therefore it was important to determine whether these fragments from the mid region of the PTH molecule can induce proliferation of bone cells *in vivo* and thus have the potential to be used to increase bone formation in pathological conditions such as osteoporosis.

MATERIALS AND METHODS

Animals

Batches of female (20–25-day-old) Wistar-derived rats (Weizmann Institute Hormone Research Departmental colony; mean weight ± S.E.M. 35 ± 1.5 g at 20 days) were divided randomly into experimental groups and injected intraperitoneally (i.p.), with doses of PTH or PTH fragments for time periods as described for each experiment. Rats were fed with pelleted chow *ad libitum* and were kept on a 14-h light/10-h dark schedule in air-conditioned rooms maintained at 23 °C. They were killed by cervical dislocation at the times indicated and organs were removed for determination of CK activity or for incubation *in vitro* with [^{3}H]thymidine for 2 h to measure DNA synthesis.

CK assay

Kidneys, tibia and femur were excised; the epiphyses or diaphyses of the long bones were collected and washed thoroughly with cold 0.9% NaCl. The bones were split open lengthwise and the marrow was scraped out. Organs were homogenized in ice-cold buffer containing 50 mM-Tris/HCl (pH 6.8), 5 mM-magnesium acetate, 2.5 mM-dithiothreitol,

Abbreviations used: hPTH, human parathyroid hormone; CK, creatine kinase; i.p., intraperitoneal.
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0.4 mM-EDTA and 250 mM-sucrose using a Polytron homogenizer in 10 s bursts with intervening cooling periods. Supernatant extracts were obtained by centrifugation at 12000 *g* for 5 min at 4 °C. CK activity was measured [34] at 30 °C in a Gilford 250 automatic recording spectrophotometer at 340 nm, using a coupled assay for ATP, in 0.5 ml of incubation mixture containing 50 mM-imidazole acetate buffer (pH 6.7), 25 mM-phosphocreatine, 20 mM-*N*-acetylcysteine, 20 mM-D-glucose, 10 mM-magnesium acetate, 5 mM-EDTA, 2 mM-ADP, 2 mM-NAD^+, 2 mM-dithiothreitol, 50 μM-diadenosine pentaphosphate (adenylate kinase inhibitor), 5 μg of bovine serum albumin, 1.2 units of glucose-6-phosphate dehydrogenase and 0.8 units of hexokinase. Protein was determined by the Coomassie Brilliant Blue dye-binding method [35], with bovine serum albumin as the standard.

[^{3}H]Thymidine incorporation into DNA

At 0, 2, 14 and 22 h after injection, the rats were killed and the tissues were removed rapidly, washed and cut into cubes, approx. 2 mm per side, which were found to give optimal incorporation. The pieces were then incubated for 2 h more (the time indicated in Fig. 6) with [^{3}H]thymidine (5 μCi/ml; 5 Ci/mmol) at 37 °C in Dulbecco's modified Eagle's medium under an atmosphere of 5% CO_2/95% air. Total [^{3}H]thymidine uptake and incorporation into trichloroacetic acid-insoluble material was measured as described previously [36]. At no time, including the 22–24 h pulse, was there any significant change in the total uptake of [^{3}H]thymidine which reflects changes in the thymidine pool which, in turn, could account for changes in incorporation. DNA was determined by the Burton method [37].

Serum Ca^{2+} determination

Blood was withdrawn from the hearts of CO_2-anaesthetized rats 24 h after injection of PTH fragments. The Ca^{2+} concentration in the serum was measured using an autoanalyser. The values obtained after injection of distilled water (0.121 ± 0.004 mg/ml; mean ± S.E.M.), hPTH-(1–34) (0.117 ± 0.005 mg/ml) and hPTH-(28–48) (0.125 ± 0.001 mg/ml) were not significantly different from each other.

Reagents

All reagents used were analytical grade. Biochemicals were obtained from Sigma (St. Louis, MO, U.S.A.). hPTH-(25–39) and hPTH-(34–47) were synthesized at the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany.

Statistical significance

The significance of differences between experimental and control values was evaluated by Student's *t* test.

RESULTS

Time- and dose-dependent stimulation of CK activity by PTH and PTH fragments

In order to determine if the activities of PTH and PTH fragments *in vitro* [32,33] were paralleled *in vivo*, prepubertal female rats were used as the model system and injected with approximately equimolar doses of PTH and PTH fragments. Both the constitutive and induced specific activities of CK in the diaphysis and epiphysis of 20-day-old female rats (> 10 μmol/min per mg of protein) were severalfold higher than the constitutive CK specific activities in kidney (which ranged between 1.5 and 4 μmol/min per mg of protein), despite the variation among constitutive specific activities in different experiments (see control and zero time values in Figures).

When 20-day-old rats were injected with 3.5 μg of bPTH-(1–84), which is equivalent to 11 nM if equally distributed throughout a 35 g rat, there was a significant increase in CK activity in the organs measured as early as 1 h after injection (Fig. 1). This increase persisted after 4 h and reached a maximum of approx. 2-fold at 24 h in diaphysis and epiphysis; in kidney at 24 h the activity had dropped to a level insignificantly greater than that at zero time. Injection with 2 μg of bPTH-(1–34)/rat, equivalent to 13 nM, caused a similar increase in CK specific activity (Fig. 2); namely, significant increases at all three times measured in the diaphysis and epiphysis, and at 1 and 24 h in kidney. The mid-portion fragment hPTH-(28–48), which has mitogenic activity *in vitro* [32,33], also increased CK activity after injection of 1.25 μg/rat, equivalent to 14 nM (Fig. 3). In kidney, CK specific activity increased to a peak between 1 and 4 h and then declined to close to the constitutive activity at 24 h. In the epiphysis there was a significant increase at 1 h, a maximal increase at 4 h and no significant increase at 24 h. However, in the diaphysis, CK activity was significantly increased at all three times measured, with the maximal increase at 1 h. Injection of rats with increasing doses of PTH-(1–34) caused a significant increase in CK specific activity 4 h later in all organs at the lowest dose tested (0.4 μg/rat) to a maximal value at 2 μg/rat, equivalent

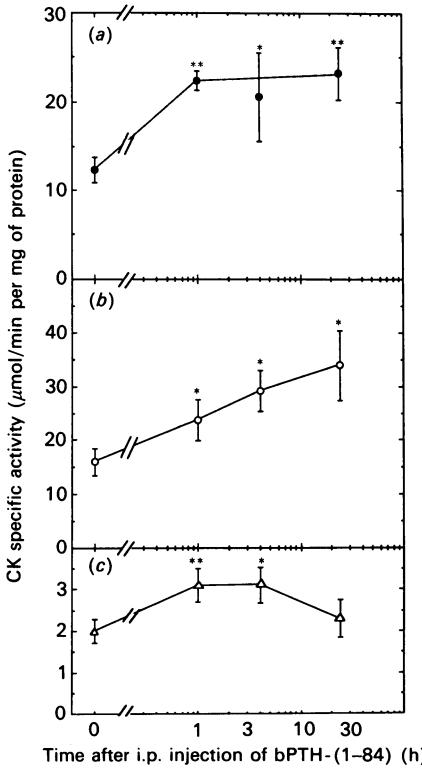

Fig. 1. Time-dependent stimulation by bPTH-(1–84) of CK specific activity in rat organs

Female rats (20–25 days old) were injected i.p. with bPTH-(1–84) (3.5 μg/rat). At the times indicated, rats were killed and the diaphyses (*a*) and epiphyses (*b*) of long bones as well as the kidneys (*c*) were collected and homogenized. Extracts were prepared and assayed as described in the Materials and methods section. Results are means ± S.E.M. for $n = 5$. Statistical significance of differences between treated and control groups by Student's *t* test: $*P < 0.05$, $**P < 0.01$. Note that the activity scales differ for different organs.

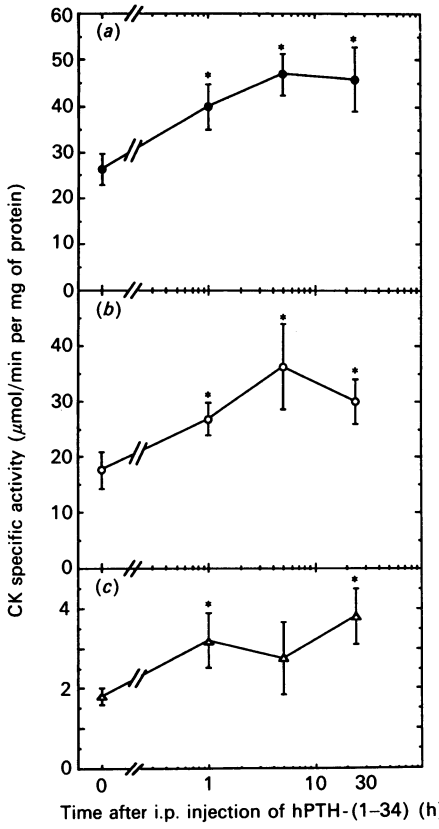

Fig. 2. Time-dependent stimulation by hPTH-(1–34) of CK specific activity in rat organs

Female rats (20–25 days old) were injected i.p. with hPTH-(1–34) (2 μg/rat). Experimental details are as described for Fig. 1. (*a*) Diaphysis, (*b*) epiphysis, (*c*) kidney. $*P < 0.05$, $**P < 0.01$ versus controls.

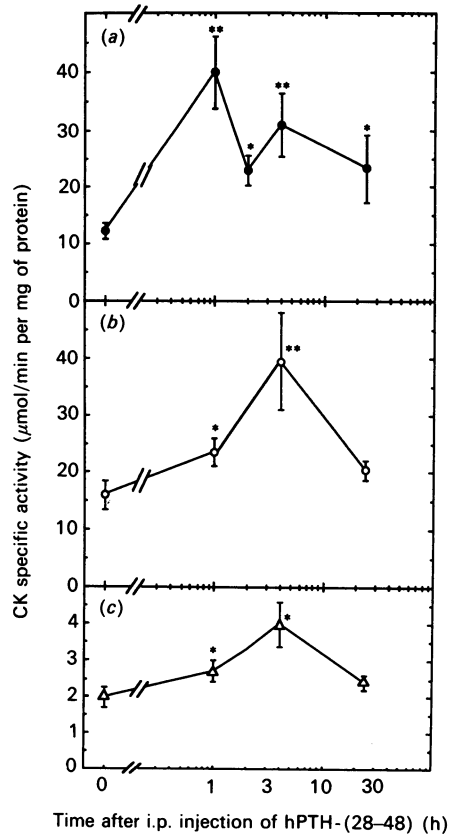

Fig. 3. Time-dependent stimulation by hPTH-(28–48) of CK specific activity in rat organs

Female rats (20–25 days old) were injected i.p. with hPTH-(28–48) (1.25 μg/rat). Experimental details are as described for Fig. 1. (*a*) Diaphysis, (*b*) epiphysis, (*c*) kidney. $*P < 0.05$, $**P < 0.01$ versus controls.

to 13 nM (Fig. 4). When rats were injected with hPTH-(28–48) and killed 4 h later, a similar result was obtained (Fig. 5); there was a significant increase in CK specific activity in all three organs at a dose of 0.15 μg/rat, with a maximum effect at 1.25 μg/rat (equivalent to 14 nM), which reached an approx. 3-fold stimulation in diaphysis.

Comparison of effects of defined PTH fragments on CK activity and DNA synthesis in rat organs

To compare the activity of PTH-(28–48) (Fig. 5) with that of other fragments derived from the mid region of PTH, rats were injected with hPTH-(25–39) or hPTH-(34–47) at a dose of 0.875 μg/rat, equivalent to 14 nM; whereas hPTH-(25–39) caused a significant increase in CK activity, hPTH-(34–47) had no effect (Table 1).

To corroborate that under the circumstances of these experiments the increase in CK specific activity in bone is indeed a valid marker of cell proliferation, changes in DNA synthesis were measured by [^{3}H]thymidine incorporation [33]. The earliest time at which PTH increased [^{3}H]thymidine incorporation was determined by injecting rats with an optimal dose of 2 μg of PTH-(1–34)/rat, equivalent to 13 nM (Fig. 4), and measuring 2 h pulses of [^{3}H]thymidine incorporation into DNA (Fig. 6). In the kidney, unlike the increase in CK activity, there was no increase in DNA synthesis at any time. In the diaphysis and the epiphysis there was no change at 4 or 16 h, but at 24 h there was a significant increase in DNA synthesis (Fig. 6). Therefore rats were injected with test compounds and 22–24 h later [^{3}H]thymidine incorporation into DNA was measured *in vitro*. hPTH-(25–39) was as potent as hPTH-(28–48) in stimulating [^{3}H]thymidine incorporation, which in turn was as potent as bPTH-(1–84) in diaphysis and epiphysis, whereas hPTH-(34–47) was inactive. In the kidney there was no significant stimulation of DNA synthesis by any of the fragments tested or by bPTH-(1–84) (Table 2), since after the age of 16 days there is negligible cell division in rat kidney, which grows by hypertrophy.

DISCUSSION

The results presented in this paper demonstrate that the mid-region fragments hPTH-(25–39) and hPTH-(28–48), as well as the full-length bPTH-(1–84), stimulate DNA synthesis in rat diaphysis and epiphysis, and CK activity in diaphysis, epiphysis and kidney. The increases are dose- and time-dependent, as shown previously using *in vitro* models of chondrocytes [32] and both osteoblast-enriched calvaria cell cultures and ROS 17/2.8 osteoblast-like cells [33]. These results are in accord with data *in vivo* for stimulation of proliferation by bPTH-(1–84) and bPTH-(1–34) in ovariectomized rats [38] and intact rats [30,38,39], dogs [4] and humans [30]. The fact that the mid-region fragments hPTH-(28–48) and hPTH-(25–39) can induce bone cell proliferation is of particular interest, since they do not stimulate cyclic AMP formation [32,33], which serves as an intracellular messenger for the osteolytic response to PTH [40]. This makes them potentially capable of promoting bone formation without

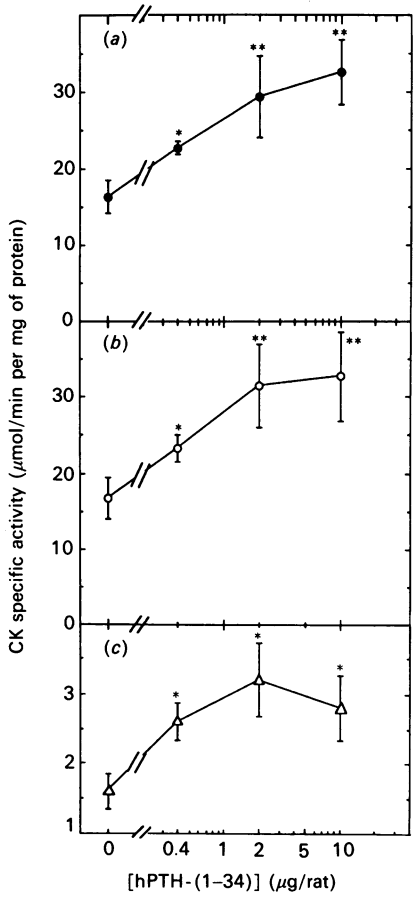

Fig. 4. Dose-dependent stimulation of CK specific activity by hPTH-(1–34) in rat organs

Female rats (20–25 days old) were injected i.p. with hPTH-(1–34). At 4 h later, rats were killed and extracts were prepared and assayed as described in the Materials and methods section. Experimental details are as described for Fig. 1. (*a*) Diaphysis, (*b*) epiphysis, (*c*) kidney. $*P < 0.05$, $**P < 0.01$ versus controls.

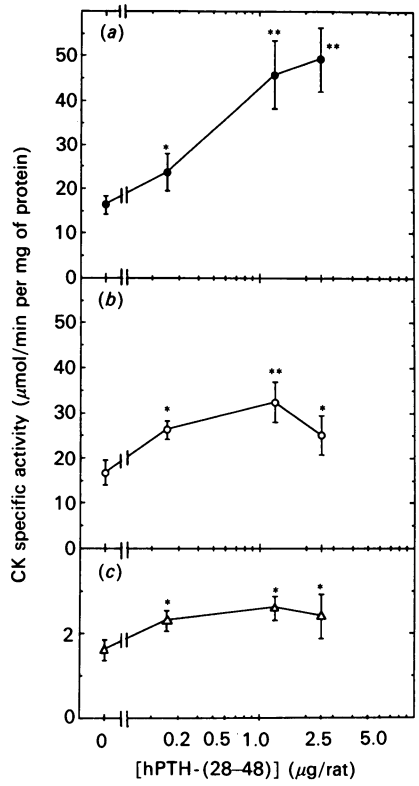

Fig. 5. Dose-dependent stimulation of CK specific activity by hPTH-(28–48) in rat organs

Female rats (20–25 days old) were injected i.p. with hPTH-(28–48). Experimental details are as described for Fig. 4. (*a*) Diaphysis, (*b*) epiphysis, (*c*) kidney. $*P < 0.05$, $**P < 0.01$ versus controls.

Table 1. Stimulation of CK specific activity by PTH fragments

CK activity was measured in rat organs 4 h after injection of 0.875 μg of hPTH-(25–39) or hPTH-(34–47) or 1.25 μg of hPTH-(28–48). Experimental details are as described for Fig. 1. * Significantly different from control; $P < 0.05$.

PTH fragment	CK specific activity (μmol/min per mg of protein)		
	Epiphysis	Diaphysis	Kidney
None	15.0±2.2	26.0±3.9	1.43±0.20
hPTH-(25–39)	24.0±3.2*	41.8±4.9*	3.22±0.78*
hPTH-(28–48)	20.9±3.0*	37.2±3.3*	2.26±0.34*
hPTH-(34–47)	16.7±2.5	24.9±4.8	1.64±0.32

bone resorption. Moreover, *in vitro* [33], hPTH-(28–48) was shown to inhibit cyclic AMP formation by bPTH-(1–84), which may further influence the equilibrium of bone turnover in the direction of formation rather than resorption. It will be of great interest to determine if fragments containing the mitogenic mid region of the PTH molecule are indeed produced and circulate *in vivo*.

The mid-region fragment hPTH-(28–48), which we utilized in this study, was originally intended as a competitive inhibitor of the cleavage of hPTH-(1–84), since it contained the major cleavage sites between residues 33 and 34 and residues 37 and 38 [41]. However, the stability of the fragment was shown by the present study to be sufficient to allow it to act as a mitogen after a single i.p. injection. The similarity between these studies *in vivo* and previous reports *in vitro* [32,33] is encouraging, since it has often been pointed out (e.g. [19]) that discrepancies between bioassays *in vitro* and *in vivo* make studies *in vivo* essential at an early stage of investigation.

The possibility of using a mid-region fragment of PTH to prevent bone loss in osteoporosis is strengthened by the recent finding [29] that resorption is not needed for stimulation of bone growth in living rats. In the present study, no significant change in serum calcium concentration was seen by 24 h after injection of a mitogenic dose of hPTH-(28–48). However, the time period of this study is not sufficient to detect significant changes in circulating Ca^{2+} concentration. Long-term investigations should provide this data, along with the histological evaluation of chronic treatment with mid-region fragments. Further studies on morphological parameters are needed as well, to see whether recent findings of favourable anabolic responses of osteoporosis patients to a combination of hPTH-(1–38) and calcitonin [42] can also be achieved by treatment with a mid-region fragment of PTH alone.

The question of whether the *N*-terminal and mid-region fragments of PTH operate via the same or different receptors ([43], and references therein) also requires further investigation. Future studies should indicate whether there is any potential for use of mid-region fragments of PTH in osteoporosis in general,

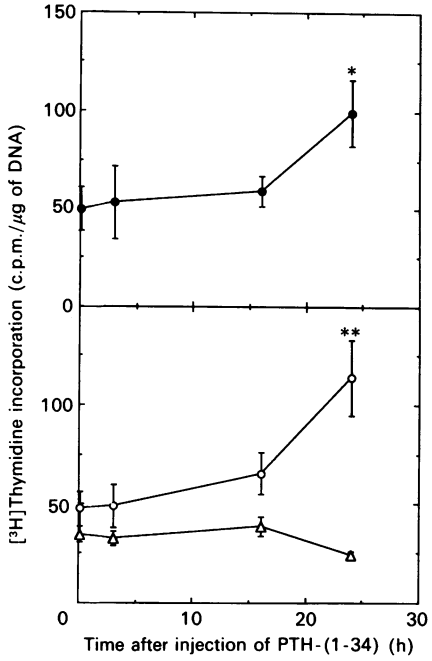

Fig. 6. Time course of stimulation by hPTH-(1–34) of DNA synthesis in rat organs

Female rats (20–25 days old) were injected i.p. with hPTH-(1–34) (2 μg/rat). At 2 h before the times indicated, the rats were killed and organs were removed and incubated in 2 ml of Dulbecco's modified Eagle's medium containing 5 μl of [^{3}H]thymidine (Amersham; 5 Ci/mmol) for 2 h, then washed with medium and homogenized in deionized distilled water. The total uptake of [^{3}H]thymidine and acid-insoluble radioactivity was measured, as well as DNA content, as described in the Materials and methods section. ●, Diaphysis; ○, epiphysis; △, kidney. $*P < 0.05$, $**P < 0.01$ versus controls.

Table 2. Stimulation of DNA synthesis by PTH fragments

DNA synthesis was measured in rat organs from 22–24 h after injection of 3.5 μg of bPTH-(1–84), 0.875 μg of hPTH-(25–39), 1.25 μg of hPTH-(28–48) or 0.875 μg of hPTH-(34–47). Experimental details are as given for Fig. 6. $*P < 0.05$, $**P < 0.01$ versus control.

PTH fragment	[^{3}H]Thymidine incorporation into DNA (c.p.m./μg of DNA)		
	Epiphysis	Diaphysis	Kidney
None	25.8±5.2	24.1±3.8	9.0±2.1
bPTH-(1–84)	40.7±3.3**	43.4±4.7**	11.8±2.4
hPTH-(25–39)	48.4±6.6*	44.8±4.5**	11.2±1.9
hPTH-(28–48)	46.7±7.4*	39.1±8.1*	11.2±2.7
hPTH-(34–47)	28.0±7.2	28.6±4.2	9.9±1.9

and in such special cases as the specific inhibition of osteoblast production during space flight ([44] and references therein).

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