Journal of Cellular Biochemistry 81:499–506 (2001)

Effect of the Neuropeptide Substance P on the Rat Bone Marrow-Derived Osteogenic Cells In Vitro

Maria A. Adamus* and Zbigniew J. Dąbrowski

Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Cracow, Poland

Abstract Substance P containing, thin, sensory nerve fibres have been demonstrated in bone and bone marrow. However the role of substance P in bone tissue is not fully understood. We investigated the effects of substance P on the growth and development of rat bone marrow-derived osteogenic cells in vitro. To examine this, the marrow-derived osteogenic cells were treated from 3rd to 6th day of subculture with substance P at concentrations 10^{-10}, 10^{-9} and 10^{-8}M. [^{3}H]-thymidine, L-2,3-[^{3}H]-proline incorporation, protein accumulation, alkaline phosphatase activity, and calcium deposition were measured in cultures. Substance P slightly stimulated [^{3}H]-thymidine incorporation at 10^{-10} M. Protein accumulation and L-2,3-[^{3}H]-proline incorporation were enhanced in a dose dependent manner. Simultaneous application of spantide, a substance P receptor antagonist, could not block substance P-induced L-2,3-[^{3}H]-proline incorporation probably because of statistically significant effect of spantide itself. Calcium deposits were significantly lower (about 30%) in cultures treated with SP. This effect was probably due in part by the fall in alkaline phosphatase activity which in substance P treated cultures was decreased about 17%. Our results indicate that substance P could be one of the factors modulating bone metabolism. J. Cell. Biochem. 81:499–506, 2001. © 2001 Wiley-Liss, Inc.

Key words: substance P; osteogenic cells; mineralization; alkaline phosphatase; dexamethasone

Substance P (SP) is an 11-amino acid neuropeptide present in thin, nonmyelinated afferent nerve fibres conducting pain and information about intense thermal, mechanical, and chemical stimuli that have the potential to cause tissue damage. The SP-positive nerve fibres are widely distributed not only in the brain and spinal cord, but also in many organs such as skin, intestine and immune system [Pernow, 1983; Bost and Pascual, 1992] usually beside the peripheral capillaries. Furthermore immunohistochemistry has revealed the presence of sensory fibres containing SP in bone tissue including bone marrow [Bjurholm et al., 1990; Bjurholm, 1991; Hukkanen et al., 1992b; Sisask et al., 1995; Goto et al., 1998]. They were particularly abundant in regions of high osteogenic activity, such as the growth plate [Hukkanen et al. 1992b]. In the course of bone fracture healing in a rat bone fracture model, the SP-positive nerve fibres were found to emerge with capillaries in newly proliferated bone tissue (callus) around the broken area [Rusanen et al., 1987; Madsen et al., 1998]. It seems therefore possible that SP can influence local metabolic processes in bone tissue.

The effect of SP on bone metabolism is still scarcely understood. Recently, this neuropeptide has been shown to stimulate bone resorption activity of rabbit osteoclasts [Mori et al., 1999]. There are also some earlier reports suggesting an influence of SP on the osteoclast function [Sherman and Chole, 1995]. The possible effect of SP on the bone formation activity of osteoblasts is unknown. Bjurholm et al. [1992] has demonstrated that SP has no effect on the cellular content of cyclic AMP in various osteoblast-like cells in vitro. This result, however, can indicate the lack of utilisation of cAMP as a second messenger but does not exclude other possible pathways of SP signalling.

Bone marrow has been recognised as the source of osteoprogenitor cells. It has been demonstrated that bone marrow stromal cells cultured in vitro, can be induced with dexamethasone to differentiate along the osteoblast lineage [Maniatopoulos et al., 1988; Leboy et al., 1991; Kamalia et al., 1992; Vilamitjana-Amedee et al., 1993; Cheng et al., 1994; Peter et al.,

Grant sponsor: BW grant from Institute of Zoology UJ.

*Correspondence to: Maria A. Adamus, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Cracow, Poland.

Received 5 July 2000; Accepted 5 October 2000

This article published online in Wiley InterScience, February XX, 2001.

1998]. They express an osteogenic phenotype and produce mineralized bone-like tissue. The aim of this study was to investigate the effect of SP on the rat bone marrow derived osteogenic cells activity in vitro.

MATERIALS AND METHODS

Materials

Foetal calf serum (FCS) was purchased from Bioproduct GmbH (Germany). α-Minimum Essential Medium (αMEM) was from Gibco, B.R.L. (Gaithersburg, MD); tissue culture dishes were purchased from Nunc (Roskilde, Denmark). Substance P, dexamethasone, ascorbic acid and penicillin were obtained from Sigma. β-Glycerophosphate was from Merck (Darmstadt, Germany). [^{3}H]-thymidine (1.08 mCi/ml) was from Chemapol (Czech Republic) and L-2,3-[^{3}H]-proline (390 mCi/mg) was purchased from Amersham Radiochemicals (Buckinghamshire, UK). All other materials used were reagent grade and were obtained from commercial sources.

Cell Culture

Bone marrow was obtained from young adult male rats of the Wistar strain, age 28–30 days and weighing 100–120 g. The femora were excised aseptically, cleaned of soft tissues and the ends of the bones were removed. Bone marrow was flushed out and the cell suspension was prepared by repeatedly aspirating the cells through the 20 gauge needle. Cells were seeded in 25 cm^2 flasks, 4×10^7 cells/flask. The non-adherent hematopoietic cells were removed after the first 2 days of culture. The cultures were grown in αMEM supplemented with 10% FCS, ascorbic acid (50 μg/ml), β-glycerophosphate (10 mM), penicilin (120 u/ml), dexamethasone (10^{-8} M). Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C and medium was changed twice a week. After 10 days in primary culture, bone marrow stromal cells were trypsinized, counted, seeded in 24-well plates at a plating density of 4×10^3 cells/cm^2 and fed the same medium. These first-passage cultures were treated with SP and served for the assays.

Confirmation of Mineralization in Cultures

Alkaline phosphatase was histochemically detected in cultures [Lojda et al., 1979]. The mineralization of the cell layer in subcultures was revealed by light microscope. The calcium deposits were further confirmed by von Kossa staining [Bellows et al., 1992] and analysed by X-ray microanalysis. To analyse the mineral deposited in cultures, first passaged cells were seeded onto 10-mm coverslips. After 5 weeks the cultures were washed three times with PBS, then fixed in 2.5% glutaraldehyde and dehydrated in graded concentrations of ethanol (30–100%, twice for 10 min at each concentration). The coverslips containing fixed cells were air dried and coated with carbon. The levels of Ca and P in densely mineralized, thick areas were analysed using scanning microscope JEOL JSM-5410 equipped with an energy dispersive spectrometer (NORAN Instruments). The accelerating voltage was 20 keV and the beam current was 1 nA.

Treatment of Cells With SP

SP and SP-receptor antagonist spantide were dissolved in endotoxin-free distilled water to a final concentration of 10^{-4} M, then were aliquoted in siliconized microcentrifuge tubes and stored at −70°C. Immediately before use SP and spantide were diluted to the appropriate concentration in serum-free culture medium. SP and spantide were used within 2–3 weeks after being solubilized.

Bone marrow-derived osteogenic cells were stimulated with SP at concentrations 10^{-10}, 10^{-9} and 10^{-8} M. The concentrations were chosen on the base of previous studies, indicating that this range of SP exerted optimal effects on bone marrow stromal cells in vitro [Rameshwar et al., 1993, 1997; Rameshwar and Gascon, 1995]. Cells were stimulated for 4 days, from the 3rd day of subculture. During the treatment period, the culture medium was changed every 24 h and SP in appropriate concentration was readded to the wells. Cultures were stopped 24 h after the last treatment. Then protein content, amount of incorporated isotopes ([^{3}H]-thymidine and L-2,3-[^{3}H]-proline) and alkaline phosphatase activity were assayed. Cultures to be assayed for the level of deposited calcium were stopped 14 days after the last SP treatment.

[^{3}H]-Thymidine Assay

Cellular proliferation was monitored by quantifying DNA synthesis through the incorporation of [^{3}H]-thymidine. Marrow-derived

osteogenic cells were treated with SP from 3rd to 6th day of subculture. On the last day of treatment, just after the last dose of SP, 1 μCi of [^{3}H]-thymidine was added to each well. After 24 h the cells were fixed with the mixture of acetic acid and methanol (3:1) for 1.5 h. Then the mixture was aspirated, the cell layer was washed twice with 80% methanol, dried and solubilized in 0.5 M NaOH (0.5 ml/well). The quantity of isotope incorporated in proliferating cells was measured in a liquid scintillation counter. Data were expressed as counts per 2 minute 5.

Protein Concentration

Effect of SP on protein synthesis in cultures was examined by studying the incorporation of L-2,3-[^{3}H]-proline. Cultures were treated with SP from 3rd to 6th day of subculture. On the last day of treatment 1 μCi of L-2,3-[^{3}H]-proline was added to each well for 24 h. Thereafter, the cultures were fixed, washed, solubilized and counted as described for [^{3}H]-thymidine incorporation. Protein in the cell layer was also measured by the Coomassie blue method of Bradford [1976], using the BSA as standard.

Alkaline Phosphatase Activity

Cultures were washed three times with PBS and scraped with a cell scraper into 250 μl of 10 mM Tris–HCl buffer pH 7.5, containing 10 mM $MgCl_2$ and 0.1% Triton X-100 and stored at −20°C about a week. Alkaline phosphatase activity was assayed in cell lysates by determining the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 37°C and pH 10.1. The substrate solution contained 7 mM *p*-nitrophenyl phosphate in glycine buffer of pH 10.1 supplemented with 2 mM $MgCl_2$. After 30 min at 37°C the reaction was stopped by 0.5 ml 0.5 M NaOH [modified after Cheng et al., 1994]. Enzyme activity was expressed as nanomoles of *p*-nitrophenol per mg protein per minute incubation time.

Deposited Calcium Level

Cultures were treated with SP or vehicle from 3rd to 6th day of subculture. Two weeks after the last treatment, the medium was removed and the cells were washed twice with PBS. Then 0.4 ml of 2M HCl was added to each well and cells were incubated for 48 h in 37°C. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations.

Specificity of SP Action

To determine the specificity of SP action, control experiments with cultures preincubated with the SP-receptor antagonist—spantide ([D-Arg^1, D-Trp^{7,9}, Leu^{11}]-substance P, [Seelig, 1990]) were performed. Cells were treated with SP at concentration 10^{-8} M for 4 days; 10 min before every SP dose, spantide (10^{-6} M) was added to the wells. Control cultures were fed medium containing spantide 10^{-6} M alone, SP 10^{-8} M alone or vehicle. Incorporation of L-2,3-[^{3}H]-proline by cultures used in this part of experiment was measured.

Statistical Analysis

All the experiments were repeated two or three times using bone marrow stromal cells obtained from different rats. Results are presented as mean ± standard deviation (SD) from 5–6 measurements. They were compared using one-way analysis of variance (ANOVA), followed by Tukey, aposteriori test except the part of experiment with antagonist spantide when two-sample analysis was applied. $P < 0.05$ was considered statistically significant.

RESULTS

Cells in Culture

Bone marrow cells in primary culture formed fibroblastic colonies, each of which was derived from a single cell of the stromal component of the bone marrow. Primary cultures reached confluence in two weeks. Secondary cultures became almost confluent by day 11 of the subculture. At days 14–15 of the subculture dense clusters of polygonal cells were formed at several places on the bottom of culture dishes. These clusters formed with time three-dimensional nodular structures that began to mineralize. The mineralized nodules could be easily identified with the naked eye. Staining with von Kossa method, specific for calcium phosphate showed these nodules to be definitely mineralized; staining for alkaline phosphatase demonstrated very high alkaline phosphatase activity in cells surrounding nodules (results not shown). Energy dispersive X-ray microanalysis showed prominent energy peaks for calcium and phosphorus, as well as secondary

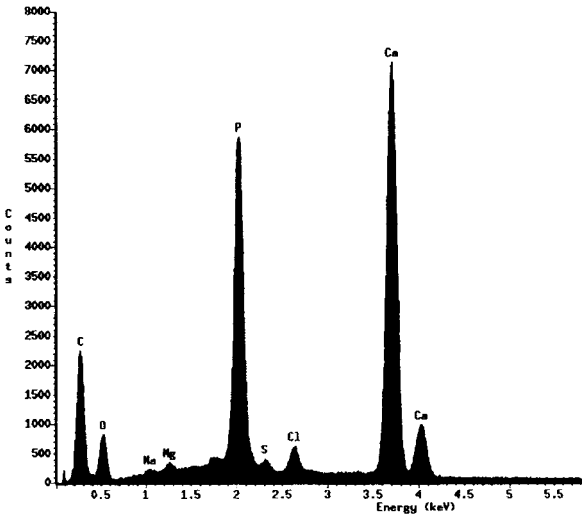

Fig. 1. Energy dispersive X-ray microanalysis spectrum showing prominent energy peaks for calcium and phosphorus.

peaks for sulphur and chlorine (Fig. 1). The Ca:P ratio was calculated:

Ca: P ratio	bone hydroxyapatite theoretical value based on the chemical formula: $Ca_5(PO_4)_3OH$	1.67
	in vitro nodules	1.70

These results suggested that the mineralized area of the nodules mainly consisted of calcium phosphate.

Effect of SP on Cell Growth and Protein Synthesis

SP added to the culture medium from 3rd to 6th day of subculture slightly enhanced marrow-derived osteogenic cell proliferation. Statistically significant ($P < 0.05$), maximal stimulation (about 19%) compared with cultures incubated without the neuropeptide was obtained at concentration 10^{-10} M SP. Higher concentrations of SP had no significant effect on marrow-derived osteogenic cell proliferation (Fig. 2).

SP stimulated protein synthesis in cultured marrow-derived osteogenic cells in a dose dependent manner (Fig. 3). Total protein content increased simultaneously with the SP concentration to reach values 24% higher than in the control group for the most potent concentration of SP—10^{-8} M.

Proline incorporation assay showed similar results. As shown in Figure 4, SP enhanced the

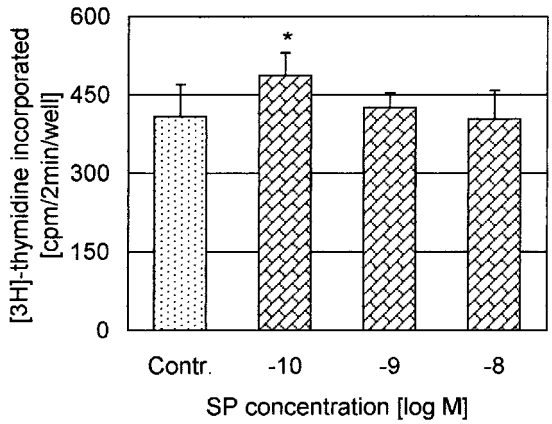

Fig. 2. Effect of substance P on [^{3}H]-thymidine incorporation in marrow-derived osteogenic cell cultures. *$P < 0.05$.

rate of L-2,3-[^{3}H]-proline incorporation in a dose dependent manner. The values obtained for 10^{-9} M and 10^{-8} M SP were respectively 23% and 28% higher than for the control cultures, the differences were statistically significant ($P < 0.05$; $P < 0.01$).

Effect of SP on the Expression of Differentiated Osteoblast Functions

The rate of Ca deposition by the marrow-derived osteogenic cell subcultures after the treatment with various concentrations of SP is shown in Figure 5. SP added to the culture medium lowered the level of mineralization. The most significant effect was observed at concentration of SP 10^{-9} M (the Ca content/well was 55% lower than in the control group).

Alkaline phosphatase is concerned as one of main factors in the mineralization process. Cells in subculture expressed high alkaline phosphatase activity (164.9 nmol/min × mg protein in the control groups). Treatment of first passaged marrow-derived osteogenic cells with SP for 4 days resulted in a decrease in the activity of the enzyme ($P < 0.01$). All applied concentrations of SP exerted similar effect; the alkaline phosphatase activity fell about 16% when compared to the control group (Fig. 6).

Specificity of the SP Action

To determine the specificity of the stimulating action of SP we performed control experiments with cultures preincubated with SP-receptor antagonist—[D-Arg^1, D-Trp^{7,9}, Leu^{11}]-substance P named spantide.

Spantide (10^{-6} M) added to the culture medium caused by itself a slight but statisti-

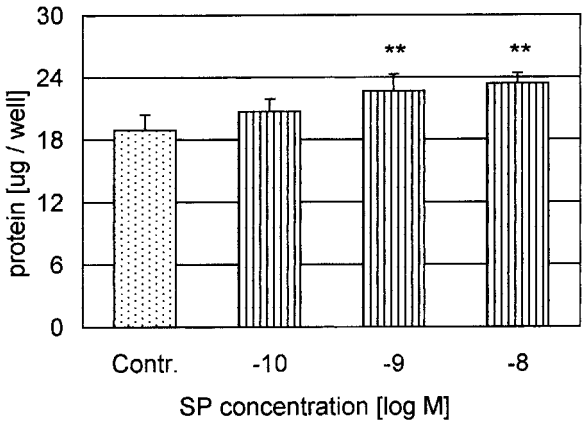

Fig. 3. Protein content in marrow-derived osteogenic cell cultures treated with substance P. $^{**}P < 0.01$.

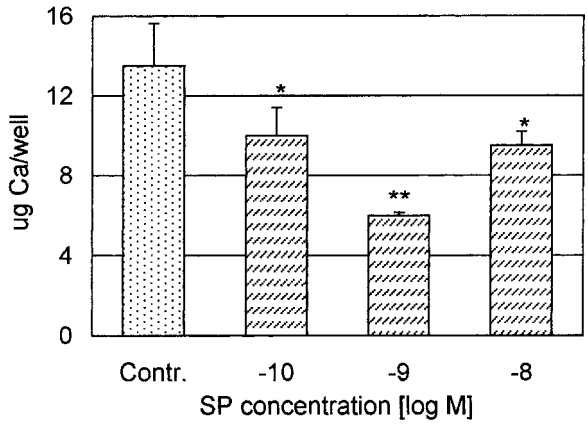

Fig. 5. Level of mineralization in marrow-derived osteogenic cell cultures treated with substance P. $^{*}P < 0.05$, $^{**}P < 0.01$.

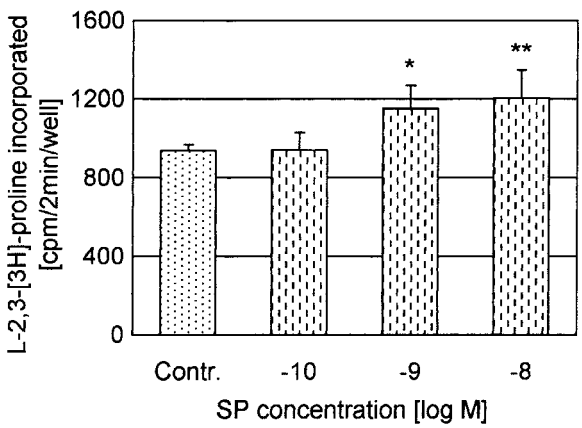

Fig. 4. Effect of substance P on L-2,3-[^{3}H]-proline incorporation in marrow-derived osteogenic cell cultures. $^{*}P < 0.05$, $^{**}P < 0.01$.

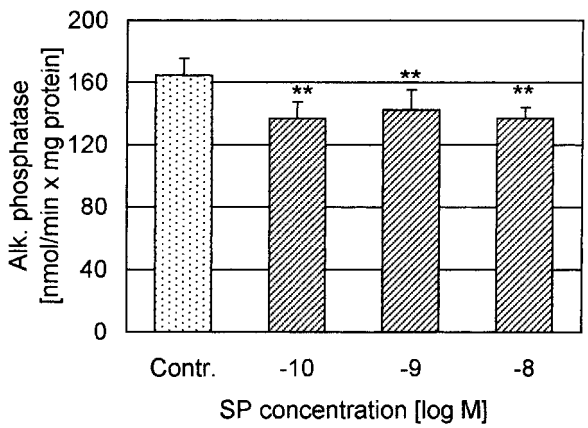

Fig. 6. Alkaline phosphatase activity in marrow-derived osteogenic cell cultures treated with substance P. $^{**}P < 0.01$.

cally significant ($P < 0.05$) stimulation of L-2,3-[^{3}H]-proline incorporation (Fig. 7). Stimulatory effect induced by 10^{-8} M SP (the concentration of SP most potent in L-2,3-[^{3}H]-proline incorporation) was slightly reduced by spantide, but the reduction was not statistically significant.

DISCUSSION

Osteoblasts and their progenitors in bone marrow may be exposed to relatively high local concentrations of SP from several sources: SP released from peripheral afferent nerve endings [Hukkanen et al., 1992a], circulating SP [Nilsson et al., 1975], SP released from endothelial cells [Ralevic et al., 1990] or macrophages [Pascual and Bost, 1990]. The role of SP in bone tissue is not exactly known. In previous experiments, chemical sympathectomy has been shown to lead to both an elevated peripheral SP [Cole et al., 1983] and increased bone resorption [Sandhu et al., 1987, 1990; Hill, 1991]. The stimulation of bone resorption has also been reported by Sherman and Chole [1995], who demonstrated that adding SP (10^{-8} M) to the culture medium induces a significant degree of calcium release from the cultured neonatal calvaria. Mori et al. [1999], has demonstrated SP to increase the intracellular calcium concentration and enhance the bone resorption activity of rabbit osteoclasts cultured in vitro. Recently capsaicin-induced sensory denervation has been shown to reduce osteoclastic resorption in adult rats [Adam et al., 2000]. According to the cited literature SP seems to influence osteoclastic activity. However, the effect of this neuropeptide on osteoblast activity has not been fully investigated yet. The

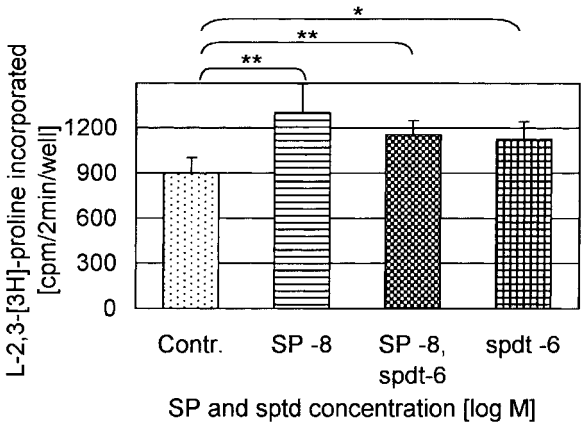

Fig. 7. Effect of substance P-receptor antagonist—spantide (spdt) on proline incorporation in marrow-derived osteogenic cell cultures. Groups were compared each with each using two-sample analysis. Three groups were found significantly different from the control group, as was shown; $*P < 0.05$, $**P < 0.01$. There was no statistically significant difference when groups (SP-8) with (SP-8 + spdt-6), (SP-8) with (spdt-6) or (SP-8 + spdt-6) with (spdt-6) were compared.

expression of SP-receptors on osteoblastic cells remains controversial. Three types of receptors for tachykinins (SP is a member of that peptide family) have been described: NK1, NK2 and NK3. SP can bind to each of the receptors, but with decreasing affinity NK1 > NK2 > NK3. Togari et al. [1997] has shown that SP-receptors mRNA is not expressed in human osteoblasts as well as in osteosarcoma cells in vitro, but the type of the SP receptor studied was not specified in that paper. Recently, Goto et al. [1998] studying the distribution of immunoreactivity for NK1-Rs in the rat bone has found numerous immunoreactive products for NK1-Rs in osteoclasts and a small number of weak, punctate products in osteoblasts and osteocytes close to the plasma membrane.

Present experiments were designed to characterise the effect of SP on growth and development of marrow-derived osteogenic cell cultures. To do this we used the culture system supporting the growth of bone marrow stromal cell population that expressed the osteogenic phenotype and produced mineralized, bone-like tissue in vitro [Maniatopoulos et al., 1988]. The light and scanning microscopic examination of cultures confirmed the high, histochemically detected, alkaline phosphatase activity and formation of a multilayered structure of cells embedded within extracellular matrix, partly mineralized by calcium phosphate.

We have demonstrated that SP can slightly stimulate the proliferation and enhance the protein synthesis in marrow-derived osteogenic cell cultures (Figs. 2–4). SP is known as a potent mitogenic factor and it has been shown to induce the proliferation of many cell types including endothelial cells [Villablanca et al., 1994], fibroblasts [Nilsson et al., 1985; Payan, 1989] and synoviocytes [Lotz et al., 1987]. Also fibroblasts from the bone marrow, when stimulated with various SP concentrations (10^{-13}–10^{-8} M) responded with more intensive proliferation [Rameshwar et al., 1997]. SP was also shown to stimulate de novo protein synthesis in fibroblasts [Kimball, 1990].

Although the exact function of skeletal alkaline phosphatase in vivo is unknown, the enzyme is thought to be involved in bone formation and calcification. In the present experiment we demonstrated that a 17% decrease in alkaline phosphatase activity coincided with fall in the level of culture calcification (Figs. 5 and 6).

Proliferation, protein synthesis, alkaline phosphatase activity and level of mineralization were determined because these markers represent the different stages of osteoblast development. Osteoblasts show a strongly regulated, sequential gene expression pattern, which consists of a reciprocal and functionally coupled relationship between proliferation and differentiation [Lian and Stein, 1995]. At the transition between proliferation and differentiation the downregulation of genes for cell proliferation and collagen synthesis coincides with the onset of expression of previously suppressed genes for matrix maturation and mineralization. Based on the findings of the current study it can be assumed that SP intensifies the proliferation phase by a prolongation of cell growth and matrix production or by inhibition of cell differentiation. This hypothesis is supported by our results: SP reduced the expression of functions typical for differentiated osteoblasts: alkaline phosphatase activity and extracellular matrix mineralization but stimulated cell proliferation and protein synthesis. It is worth to notice that the effect of SP on the proliferation and protein production was opposite and dependent on the neuropeptide concentration. Low concentration (10^{-10} M) enhanced cell proliferation but did not change the protein synthesis; higher concentrations (10^{-9} M, 10^{-8} M) significantly supported pro-

tein production but did not influence the cell proliferation. It may reflect the fact of linkage of extracellular matrix formation induction to the downregulation of proliferation and suggest that concentration of SP (or factors induced by the neuropeptide) may give the signal to go to determined step in cell developmental sequence.

To examine the specificity of SP action we determined the response of marrow-derived osteogenic cells stimulated with the highest SP concentration (10^{-8}M) to spantide, a pantachykinin antagonist [Regoli, 1989] (Fig. 7). Spantide slightly reduced the activation of L-2,3-[^{3}H]-proline incorporation elicited by SP suggesting a competitive inhibition of receptor binding, but was unable to completely block it to background levels. It could be probably explained by the fact, that spantide alone had a statistically significant stimulatory effect, consistent with the previously reported agonistic activity of the SP analogues [Pernow, 1983].

In the present study it was shown that SP has the capacity to stimulate both the growth and protein synthesis of marrow-derived osteogenic cells. This coincided with the decreased capacity of the treated cells to produce bone-like tissue in culture. Since bone tissue is innervated by fibres containing SP, it is suggested that this neuropeptide may affect bone growth and remodelling in vivo, acting together with other regulating factors.

ACKNOWLEDGMENTS

The Authors thank Mrs. Ewa Gansiniec for taking care of animals and Ms. Urszula Klaput for technical assistance.

REFERENCES

Adam C, Llorens A, Baroukh B, Cherruau M, Saffar JL. 2000. Effects of capsaicin-induced sensory denervation on osteoclastic resorption in adult rats. Exp Physiol 85: 61–66.

Bellows CG, Heersche JNM, Aubin JE. 1992. Inorganic phosphate added exogenously or released from β-glycerophosphate initiates mineralization of osteoid nodules in vitro. Bone Miner 17:15–29.

Bjurholm A. 1991. Neuroendocrine peptides in bone. Int Orthop 15:325–329.

Bjurholm A, Kreicbergs A, Dahlberg L, Schultzberg M. 1990. The occurrence of neuropeptides at different stages of DBM-induced heterotopic bone formation. Bone Miner 10:95–107.

Bjurholm A, Kreicbergs A, Schultzberg M, Lerner UH. 1992. Neuroendocrine regulation of cyclic AMP formation in osteoblastic cell lines (UMR-106-01, ROS 17/2.8, MC3T3-E1 and Saos-2) and primary bone cells. J Bone Min Res 7:1011–1019.

Bost KL, Pascual DW. 1992. Substance P: a late-acting B lymphocyte differentiation cofactor. Am J Physiol 262: 537–545.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Cole DF, Bloom SR, Burnstock G, Butler JM, McGregor GP, Saffery MJ, Unger WG, Zhang SQ. 1983. Increase in SP-like immunoreactivity in nerve fibers of rabbit iris and ciliary body one to four months following sympathetic denervation. Exp Eye Res 37:191–197.

Goto T, Yamaza T, Kido MA, Tanaka T. 1998. Light- and electron-microscopic study of the distribution of axons containing substance P and the localization of neurokinin-1 receptor in bone. Cell Tissue Res 293:87–93.

Hill EL, Turner R, Elde R. 1991. Effects of neonatal sympathectomy and capsaicin treatment on bone remodeling in rats. Neurosci 44:747–755.

Hukkanen M, Konttinen YT, Rees RG, Gibson SJ, Santavirta S, Polak JM. 1992a. Innervation of bone from healthy and arthritic rats by substance P and calcitonin gene related peptide containing sensory fibers. J Rheumatol 19:1252–1259.

Hukkanen M, Konttinen YT, Rees RG, Santavirta S, Terenghi G, Polak JM. 1992b. Distribution of nerve endings and sensory neuropeptides in rat synovium, meniscus and bone. Int J Tissue React XIV:1–10.

Kamalia N, McCulloch CAG, Tanebaum HC, Limeback H. 1992. Dexamethasone recruitment of self-renewing osteoprogenitor cells in chick bone marrow stromal cell cultures. Blood 79:320–326.

Kimball ES. 1990. Substance P, cytokines and arthritis. Ann NY Acad Sci 594:293–308.

Leboy PS, Beresford JN, Devlin C, Owen ME. 1991. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.

Lian JB, Stein GS. 1995. Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. Iowa Orthop J 15:118–140.

Lojda Z., Gossrau R., Schiebler T.H. 1979. Enzyme Histochemistry. A Laboratory Manual. Berlin-Heidelberg-New York: Springer-Verlag. 64 p.

Lotz M, Carson DA, Vaughau JH. 1987. Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. Science 235:893–895.

Madsen JE, Hukkanen M, Aune AK, Basran I, Moller JF, Polak JM, Nordsletten L. 1998. Fracture healing and callus innervation after peripheral nerve resection in rats. Clin Orthop 351:230–240.

Maniatopoulos C, Sodek J, Melcher AH. 1988. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tiss Res 254:317–330.

Mori T, Ogata T, Okumura H, Shibata T, Nakamura Y, Kataoka K. 1999. Substance P regulates the function of rabbit cultured osteoclast; increase of intracellular free calcium concentration and enhancement of bone resorption. Biochem Biophys Res Comm 262:418–422.

Nilsson G, Pernow B, Fisher GH, Folkers K. 1975. Presence of substance P-like immunoreactivity in plasma from man and dog. Acta Physiol Scand 94:542–544.

Nilsson J, von Euler AM, Dalsgaard C-J. 1985. Stimulation of connective tissue cell growth by substance P and substance K. Nature 315:61–63.

Pascual DW, Bost KL. 1990. Substance P production by P388D1 macrophages: a possible autocrine function for this neuropeptide. Immunology 71:52–58.

Payan DG. 1989. Neuropeptides and inflammation: the role of substance P. Annu Rev Med 40:341–357.

Pernow B. 1983. Substance P. Pharmac Rev 35:85–141.

Peter SJ, Liang CR, Kim DJ, Widmer MS, Mikos AG. 1998. Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, β-glycerophosphate and L-ascorbic acid. J Cell Biochem 71:55–62.

Ralevic V, Milner P, Hudlicka O, Kristek F, Burnstock G. 1990. Substance P is released from the endothelium of normal and capsaicin-treated rat hind-limb vasculature, in vivo, by increased flow. Circ Res 65:1178–1184.

Rameshwar P, Gascon P. 1995. Substance P mediates production of stem cell factor and interleukin-1 in bone marrow stroma: potential autoregulatory role for these cytokines in SP receptor expression and induction. Blood 86:482–490.

Rameshwar P, Ganea D, Gascón P. 1993. In vitro stimulatory effect of substance P on hematopoiesis. Blood 81:391–398.

Rameshwar P, Poddar A, Zhu GF, Gascon P. 1997. Receptor induction regulates the synergistic effects of substance P with IL-1 and platelet-derived growth factor on the proliferation of bone marrow fibroblasts. J Immunol 158: 3417–3424.

Regoli D, Drapeau G, Dion S, D'Orleans-Juste P. 1989. Receptors for substance P and related neurokinins. Pharmacology 38:1–9.

Rusanen M, Korkala O, Gronblad M, Partanen S, Nederstrom A. 1987. Evolution of substance P immunofluorescent nerves in callus tissue during fracture healing. J Trauma 27:1340–1343.

Sandhu HS, Herskovits, Singh IJ. 1987. Effect of surgical sympathectomy on bone remodeling at rat incisor and molar root sockets. Anat Rec 219:32–38.

Sandhu HS, Kwong-hing A, Herskovits, Singh IJ.1990. The early effects of surgical sympathectomy on bone resorption in the rat incisor socket. Arch Oral Biol 35:1003–1007.

Seelig A. 1990. Substance P and antagonists. Surface activity and molecular shapes. Biochem Biophys Acta 1030:111–118.

Sherman BE, Chole RA. 1995. A mechanism for sympathectomy-induced bone resorption in the middle ear. Otolaryngol Head Neck Surg 113:569–581.

Sisask G, Bjurholm A, Ahmed M, Keicbergs A. 1995. Ontogeny of sensory nerves in the developing skeleton. Anatom Rec 243:234–240.

Togari A, Arai M, Mizutani S, Mizutani S, Koshihara Y, Nagatsu T. 1997. Expression of mRNAs for neuropeptide receptors and β-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. Neurosci Lett 233:125–128.

Vilamitjana-Amedee J, Bareille R, Rouais F, Caplan AI, Harmand M-F. 1993. Human bone marrow stromal cells express an osteoblastic phenotype in culture. In Vitro Cell Dev Biol 29A:699–707.

Villablanca AC, Murphy CJ, Reid TW. 1994. Growth-promoting effects of substance P on endothelial cells in vitro. Synergism with calcitonin gene-related peptide, insulin and plasma factors. Circ Res 75: 1113–1120.