Expression of Serotonin Receptors in Bone*

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The 5-hydroxytryptamine (5-HT) receptors 5-HT2A, 5-HT2B, and 5-HT2C belong to a subfamily of serotonin receptors. Amino acid and mRNA sequences of these receptors have been published for several species including man. The 5-HT2 receptors have been reported to act on nervous, muscle, and endothelial tissues. Here we report the presence of 5-HT2B receptor in fetal chicken bone cells. 5-HT2B receptor mRNA expression was demonstrated in osteocytes, osteoblasts, and periosteal fibroblasts, a population containing osteoblast precursor cells. Pharmacological studies using several agonists and antagonists showed that occupancy of the 5-HT2B receptor stimulates the proliferation of periosteal fibroblasts. Activity of the 5-HT2A receptor could however not be excluded. mRNA for both receptors was shown to be equally present in adult mouse osteoblasts. Osteocytes, which showed the highest expression of 5-HT2B receptor mRNA in chicken, and to a lesser extent osteoblasts, are considered to be mechanosensor cells involved in the adaptation of bone to its mechanical usage. Nitric oxide is one of the signaling molecules that is released upon mechanical stimulation of osteocytes and osteoblasts. The serotonin analog a-methyl-5-HT, which preferentially binds to 5-HT2 receptors, decreased nitric oxide release by mechanically stimulated mouse osteoblasts. These results demonstrate that serotonin is involved in bone metabolism and its mechanoregulation.

The main highly differentiated cell types in bone tissue are the osteoclast, the osteoblast (OB), and the osteocyte (OCY). Osteoclasts are multinucleated cells of hemopoietic origin and are related to the monocyte/macrophage (1, 2, 3). Osteoblasts on the other hand are of mesenchymal stock. Their stem and progenitor cells are present in the periosteum, endosteum, and bone marrow stroma (4). Osteocytes differentiate from osteoblasts. During the process of bone formation, a number of cells from the osteoblast layer is encapsulated in the newly formed bone matrix, whereas the other cells retrace with the bone formation front. The cells that are incorporated in the bone matrix change from a cuboidal into a more stellate-shaped morphology and become osteocytes. The result of this process is a three-dimensional cellular network of osteocytes within the mineralized matrix connected to each other and to the bone surface cells by thin cell processes (5).

Together these three cell types produce, maintain, and adapt or repair, if necessary, the mineralized bone matrix. Osteoclasts are capable of bone resorption, osteoblasts of bone formation. The function of osteocytes is less well understood. Because of their location within the bone matrix, osteocytes are in a prime position for the detection of mechanical inadequacies of the tissue. They are believed to act as sensor cells that translate mechanical stimuli resulting from gravitational and muscular forces on the skeleton into biochemical signals. These biochemical signals in turn activate the effectors of bone turnover, osteoclasts and osteoblasts, leading to adaptation of mass and structure of bone (6). Indeed, both in vivo and in vitro, osteocytes were shown to be very sensitive to mechanical perturbation and to produce prostaglandins and nitric oxide upon mechanical stimulation (7–11). These locally produced factors have been reported to be involved in the adaptation of bone to its mechanical environment in vivo (12, 13).

The activities of the osteoclasts, osteoblasts, and osteocytes, as well as proliferation and differentiation of their precursor cells, are regulated by a multitude of factors. Some of these factors are of humoral origin (hormones), whereas others are locally produced (growth factors and cytokines), often in response to hormonal or mechanical activation. Apart from the clearly bone-related classical hormones (parathyroid hormone, 1,25-dihydroxyvitamin D3, estrogen, calcitonin) and well established or recently discovered local factors (insulin-like growth factors, transforming growth factors, prostaglandins, osteoclast differentiation factor, bone morphogenetic proteins, and others), a number of neurotransmitters that seem unlikely to be related to bone metabolism have been found to play a role in bone (14, 15). The presence of functional receptors for vasoac-

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entially (although not exclusively), expressed in osteocytes proved to be the serotonin receptor subtype 2B (5-hydroxytryptamine 2B; 5-HT2B).

The serotonin (5-hydroxytryptamine or 5-HT) receptor family is divided into seven subfamilies (5-HT1-7 receptors), each of which is further subdivided into a number of subtypes (22). Except for the 5-HT1A receptor, serotonin receptors belong to the G-protein-coupled receptor superfamily, which is characterized by seven membrane-spanning hydrophobic regions, an N-terminal extracellular and a C-terminal intracellular domain. The 5-HT3 receptor subfamily consists of three members, i.e., 5-HT2A, 5-HT2B, and 5-HT2C (previously designated as 5-HT1C) receptors. The 5-HT2B receptor has been detected in many peripheral organs in several mammalian species including humans, primarily in the cardiovascular system, the gut, and the stomach fundus. Its expression level in the brain is, however, generally found to be relatively low compared with that of most other 5-HT receptors (23–29). The 5-HT2B receptor has not yet been described in birds, nor in bone or bone cells.

In this work, we have studied the mRNA expression of the 5-HT2B receptor in bone and other tissues of 18-day-old fetal chickens and compared its expression in isolated osteocytes with the expression in isolated osteoblasts and periosteal fibroblasts (PF) isolated from the outer periosteum. This last population consists of mesenchymal cells with a fibroblast-like morphology in cell culture. The outer periosteum contains osteoblast precursor cells as is shown by their capacity for bone matrix formation (30). To show that not only 5-HT2B receptor mRNA but also 5-HT2B receptor protein is present in bone cells, we have examined the effects of α-methyl-5-HT, a 5-HT analog, which preferentially binds to 5-HT2 receptors (31), on the proliferation of periosteal fibroblasts. A number of specific agonists and antagonists were used in pharmacological studies to investigate whether 5-HT2B receptors are involved in the regulation of cell proliferation. Finally, we have studied the expression of serotonin receptors in isolated mouse osteoblasts to demonstrate that the expression of 5-HT2 receptors in bone is not restricted to chicken. In addition, mouse osteoblasts were used to study possible effects of serotonin in the response of bone cells to mechanical loading. Although osteocytes are the pivotal cells in the mechanoregulation of bone, (mouse) osteoblasts have also been shown to be mechanosensitive (32). Since osteocytes are extremely difficult to isolate in adequate numbers (33), mouse osteoblasts were used in a preliminary study to examine whether serotonin could modulate the mechanosensitivity of bone cells. If serotonin does modulate the mechanosensitivity of bone cells, this could justify the relatively high expression of 5-HT2B receptor in osteocytes, the mechanosensory cells per excellence.

MATERIALS AND METHODS

Tissue Dissection and Cell Isolation—Various tissues (skin, heart, liver, brain, intestine, skeletal muscle, and both calvarial and long bone) were dissected from 18-day-old fetal chickens, immediately put into RNAzol (CAMPRO Scientific, Veenendaal, The Netherlands), cut into small pieces and homogenized for the isolation of RNA according to the manufacturer’s protocol.

Various bone cell types were isolated from fetal chicken calvarial bones as described (33). In short, calvariae were aseptically dissected and periosteum were removed. A mixed population of primarily osteoblasts and osteocytes (OBmix) was obtained from the calvariae by sequential treatments with collagenase (1 mg/ml; Sigma, Zwijndrecht, The Netherlands) and EDTA (4 min). The OBmix population was cultured in α-minimal essential medium (αMEM) (Life Technologies, Inc., Breda, The Netherlands), supplemented with 200 μg/ml glutamine (Sigma), 50 μg/ml gentamycin sulfate (Sigma), 50 μg/ml l-ascorbic acid (BDH,布鲁士威奇化学,阿姆斯特丹,荷兰), 1 mg/ml α-glucose (BDH) (complete medium), and 2% chicken serum (Life Technologies, Inc.). After 1 day of culture, the OBmix cells were harvested by 3 min of trypsin/EDTA treatment (0.05%/0.01% in phosphate-buffered saline) at 37 °C. Osteoblasts (OB) and osteocytes (OCY) were separated from each other by immunomagnetic separation, using monoclonal antibody MAb OB 7.3 (34). Briefly, the OBmix cell suspension was filtered through a nylon filter (pore size, 30 μm). The resulting single cell suspension was incubated with MAb OB 7.3 bound to magnetic beads with a DNA linker (Dynal, MAB OB 7.3 biotin). The resulting beads present on OCY only. The cell suspension was placed in a magnetic field, which attracted the bead-bound cells to one side of the tube. The non-bound cells (OB) were removed, leaving only the bead-bound OCY in the tube. The OB population was treated for a second time with MAb OB 7.3 bound to magnetic beads to remove the last remaining OCY. The prepared OB population, consisting of mesenchymal cells with fibroblast-like morphology in cell culture, the outer periosteum contains osteoblast precursor cells as is shown by their capacity for bone matrix formation (30). To show that not only 5-HT2B receptor mRNA but also 5-HT2B receptor protein is present in bone cells, we have examined the effects of α-methyl-5-HT, a 5-HT analog, which preferentially binds to 5-HT2 receptors (31), on the proliferation of periosteal fibroblasts. A number of specific agonists and antagonists were used in pharmacological studies to investigate whether 5-HT2B receptors are involved in the regulation of cell proliferation. Finally, we have studied the expression of serotonin receptors in isolated mouse osteoblasts to demonstrate that the expression of 5-HT2 receptors in bone is not restricted to chicken. In addition, mouse osteoblasts were used to study possible effects of serotonin in the response of bone cells to mechanical loading. Although osteocytes are the pivotal cells in the mechanoregulation of bone, (mouse) osteoblasts have also been shown to be mechanosensitive (32). Since osteocytes are extremely difficult to isolate in adequate numbers (33), mouse osteoblasts were used in a preliminary study to examine whether serotonin could modulate the mechanosensitivity of bone cells. If serotonin does modulate the mechanosensitivity of bone cells, this could justify the relatively high expression of 5-HT2B receptor in osteocytes, the mechanosensory cells per excellence.

Suppression Subtractive Hybridization—The PCR-Select cDNA Subtraction™ kit (CLONTECH, Heidelberg, Germany) allows the isolation of genes that are expressed to a higher extent in one cell type compared with another cell type (suppression subtractive hybridization; Ref. 21) and was used according to the manufacturer’s instructions to compare gene expression in isolated chicken osteocytes to that in osteoblasts. In short, cDNA was amplified from total RNA of both cell populations, using the SMART™ cDNA Amplification Kit (CLONTECH). The resulting cDNA samples were digested with Real. Subsequently, the OCY cDNA was subcloned into two portions, and each was ligated to a different double-stranded cDNA adaptor. The ends of the adaptors lacked a phosphate group, so only one strand of each adaptor was attached to the 5'-ends of the cDNAs. In the first hybridization step, an excess of OB cDNA was added to each sample of OCY cDNA, the samples were heat-denatured and allowed to hybridize for 6 h at 68 °C. Hereafter, the two primary hybridization samples were mixed, a fresh aliquot of denatured OB cDNA was added, and the samples were allowed to hybridize for 6 h. During hybridization, so-called single-copy cDNA hybrids were formed (21), but only hybrids containing two different adaptors, could be amplified by suppression PCR. These fragments represent genes that are differentially expressed in OCY. The resulting subtracted cDNA pool was cloned using the TOPO™TA Cloning Kit (Invitrogen, Groningen, The Netherlands).

RT-PCR—Total RNA was extracted from cells and homogenized tissues with RNAzol B (CAMPRO Scientific) according to the manufacturer’s instructions. For cDNA synthesis, 2 μl of random hexamers (0.1 μg/μl; Promega, Leiden, The Netherlands) was added to 10-μl RNA samples, which were incubated for 10 min at 72 °C and then cooled to 4 °C. Subsequently, 200 units of M-MLV reverse transcriptase (Promega), 10 μM dithiothreitol (Promega), 0.5 μM dNTPs (Amer sham Pharmacia Biotech, Rosendaal, The Netherlands) and 1× first strand buffer (Promega) were added to each sample. Samples were incubated at 1 h at 37 °C, after which the reaction was terminated by incubating 5 min at 95 °C. To check for genomic DNA contamination, controls were incorporated by omitting the reverse transcriptase.

PCR reactions were performed using specific primer sets (See Table I). All primer combinations were designed with the Primer3™ program of the University of Florida Sequencing Facility, Power-User Group, Inc. Madison, WI). The primer set for chicken 5-HT2B receptor was selected from the sequence obtained in this study, the others from published sequences (Table I). PCR reactions were performed using serial dilutions of 1:30, 1:90, and 1:270 of the original cDNA samples (36) in a thermal cycler (Perkin-Elmer 2400). PCR reactions contained 1× PCR-buffer (Perkin-Elmer Life Sciences), 1.5 mM MgCl2, 0.4 mM...


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TABLE I

| Gene          | Primer 1  | Primer 2  | Product length | GenBank™ Acc. No | Position |
|---------------|-----------|-----------|----------------|-----------------|----------|
| cGAPDH        | 5'-CAC GCC ACT ACT TTC C-3'  | 5'-CAC AAT GCC AAA GTT GTC-3'  | bp              | K01458         | 300°     |
| c5-HT<sub>2</sub>B<br> | 5'-GAT CAA CAA GCC ACC TCA AC-3' | 5'-ATA CCT GCC GAA AGC CTC C-3' | 420             | AF217225       | 563-546  |
| m5-HT<sub>2</sub>B<br> | 5'-CTC TTT TCA ACT GCC TCC ACT-3' | 5'-CCA GCA TTG CCA CTT TTT C-3' | 445             | NM_005811      | 254-273  |
| m5-HT<sub>2</sub>A<br> | 5'-TTG AGG AAG AAG CCA CGT-3' | 5'-CCT TGT ACT GCC AAT TG-3' | 420             | S49542         | 674-656  |
| m5-HT<sub>2</sub>A<br> | 5'-CTG AGG GAC GAA AGC AAA G-3' | 5'-CAC ATA GCC AAT CAA AAA C-3' | 498             | X72230         | 1135-1153 |

* c, chicken; m, mouse.

Length of PCR product on genomic DNA is 656 bp (Acc. No. M11213).

dNTPs, 0.2 mm of each primer, and 0.5 U AmpliTag (PerkinElmer Life Sciences). Samples were denatured for 5 min at 94 °C, followed by 35 cycles of 30-s denaturing at 94 °C, 30 s at the annealing temperature indicated in Table I, and a 30-s extension at 72 °C. A final extension of 7 min at 72 °C concluded the PCR. Intron-spanning GAPDH-specific primers were used to equalize the starting amounts of cDNA and to check for possible genomic DNA contamination. PCR fragments were analyzed by gel electrophoresis on a 1.6% agarose gel in 0.5× Tris/ Borate/EDTA containing ethidium bromide. The intensity of each band was analyzed using image analysis software (Eastman Kodak Digital Science, ID Image Analysis).

**Northern Blot Analysis**—Total RNA samples from various tissues and cells were electrophoresed on a 1% agarose gel in 1× Tris/Borate/EDTA containing 5 mM guanidine thiocyanate (37), transferred to Hybond membranes (Amersham Pharmacia Biotech) and hybridized overnight at 55 °C with a-[32P]dCTP-labeled cDNA probes in NaP/SDS hybridization buffer (0.4 mM NaH2PO4, 0.1 mM Na2HPO4, 0.7% SDS, 1× SSC, 1 mM EDTA). Probes were made by PCR using the appropriate primer pairs (Table I), electrophoresed, extracted from gel by QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK) and labeled using Ready-to-go DNA-labeling beads (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Unincorporated nucleotides were removed with ProbeQuantG-50 Micro Columns (Amersham Pharmacia Biotech). Next day, the blots were washed 2–3 times for 5 min in 2× SSC, 0.1% SDS at 60 °C. Northern blots were then exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) screen, scanned the next day, and net intensities were determined using ImageQuant software (Molecular Dynamics). Loading of RNA was assessed with a GAPDH probe. Autoradiographs of the blots were obtained by exposure to Fuji Medical x-ray film at ~80 °C.

**Alkaline Phosphatase Activity**—Chicken OB were seeded at various cell densities in 24-well plates in complete medium (see “Materials and Methods”) and cultured for 1 day to attach. Then the cells were cultured for 1, 2, or 3 days in complete medium containing either 2% chicken serum or 0.25% bovine serum albumin (BSA Fraction V; ICN Biomedical Inc., Zoetermeer, The Netherlands), and α-methyl-5-HT (ICN Biomedical Inc.) in a concentration range of 0–10−5 M (dissolved in medium). Cultures were terminated by washing the cell layers with cMEM and lysing the cells with CyQuant lysis buffer (Molecular Probes, Leiden, The Netherlands). DNA content was measured using the CyQuant cell proliferation assay kit (Molecular Probes). Alkaline phosphatase activity was measured in the same cell lysates using the method of Bessey et al. (38). In this method alkaline phosphatase activity is expressed as the amount of p-nitrophenol formed from p-nitrophenylphosphate under alkaline conditions.

**Cell Proliferation**—Chicken PF were suspended in complete medium with 2% chicken serum (10° cells/ml) and seeded into a Petri dish containing multiwell slides (10 wells per slide; ICN Biomedical Inc.). The cells were allowed to attach overnight. Then the medium was replaced by complete medium, containing 0.1% serum. After 2 days of culture, the medium was exchanged for fresh complete medium in which all sera was substituted by 0.25% bovine serum albumin to synchronize the cells and prevent down-regulation of receptors by serotonin in serum (39). The factors to be studied were added to this medium. The cell culture was continued for another 24 h. Two hours before termination of the culture bromodeoxyuridine (BrdUrd; Aldrich Chem. Co.) was added in a final concentration of 3 × 10−5 M. After termination of the culture period, the cells were fixed with zinc fixative (40), and BrdUrd incorporated in the DNA was visualized with anti-BrdUrd monoclonal antibody MoBu-1 (41), followed by horseradish peroxidase conjugated to rabbit anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands), and NovaRED (Vector Laboratories Inc., Brunschwig, Amsterdam, The Netherlands) as substrate according to standard procedures. Proliferation was expressed as the percentage BrdUrd-positive cells.

The following compounds were dissolved according to the manufacturer’s instructions and tested for their effects on PF proliferation: the 5-HT<sub>2a</sub> receptor agonist 5-hydroxy-5-HT (ICN Biomedical Inc.), the 5-HT<sub>2b</sub> receptor selective agonist BW245C86 (RBI-Sigma), and selective antagonists ketanserin (5-HT<sub>2a</sub> and 5-HT<sub>2c</sub>; RBI-Sigma), SB205653 (5-HT<sub>2a</sub> and 5-HT<sub>2c</sub>; RBI-Sigma) and SB215505 (5-HT<sub>2b</sub>; a generous gift from Dr. M. S. Duxoo, SmithKline Beecham). Controls were incorporated by adding only appropriate vehicle.

**Pulsating Fluid Flow (PFF)** and Nitric Oxide—Subconfluent mouse osteoblast cultures were trypsinized with 0.25% trypsin in phosphate-buffered saline. The cells were seeded onto polyluysine-coated glass slides at 5 × 10° cells/slide, cultured for 1 day in DMEM with 2% fetal bovine serum, and then incubated for 1 h in the presence or absence of PFF at 37 °C and 5% CO2. PFF was generated as described before (32). In short, 15 ml of culture medium was pumped through a parallel-plate flow chamber in a pulsating manner. This resulted in a pulsating (5 Hz) fluid shear stress of 0.7 ± 0.03 Pascal and an estimated peak stress rate of 12 Pascal/s. A glass slide on which osteoblasts had been seeded served as the bottom of the chamber. At 5, 10, 30, and 60 min, samples of 0.5 ml were taken from the PFF medium and the stationary control cultures. In these samples NO release was measured as NO2 using Griess reagent (40, 42). After 60 min, the osteoblasts of PFF and control slides were lysed with CyQuant lysis buffer, and DNA contents were measured using the CyQuant cell proliferation assay kit.

**Statistical Analysis**—All data were tested for normality with Shapiro-Wilk. Normality was assumed at p values higher than 0.05. Non-normally distributed data were analyzed for overall differences with a univariate general linear model (GLM), significance was assumed at p values lower than 0.05. When differences were present, Mann-Whitney U tests with Bonferroni correction for repeated testing were used to determine individual differences.

**RESULTS**

The suppression subtractive hybridization method we used to compare gene expression in isolated chicken osteocytes (OCY) and osteoblasts (OB) resulted in a large number of clones. Sequence analysis revealed that about one-third of the clones showed homology to known genes. These results will be published elsewhere.

The nucleotide sequence of one of the clones consisted of a stretch of 816 bp (GenBank™ accession number AF217225), which showed 72% identity with human 5-hydroxytryptamine receptor 2B (5-HT<sub>2b</sub>) mRNA, and 69% identity with murine 5-HT<sub>2b</sub> receptor mRNA (BLAST 2 sequence). The translated peptide sequence (AAP20211), consisting of 271 amino acids (translated with BLAST), showed 74% identity and 82% homology to the human and mouse 5-HT<sub>2b</sub> receptor amino acid sequence, respectively (Fig. 1). The sequence described here, obviously part of the chicken 5-HT<sub>2b</sub> receptor, represents the four C-terminal transmembrane domains of the 5-HT<sub>2b</sub> recep-
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The fact that we obtained the clone by using suppression subtractive hybridization implies that the receptor is expressed to a higher extent in OCY than in OB. This was confirmed by semiquantitative RT-PCR experiments (Fig. 2). In these experiments, the 5-HT_{2B} receptor and GAPDH cDNA were amplified simultaneously in one reaction, with an annealing temperature of 53°C. The reliability of these simultaneous reactions was first validated, by comparing the results with separate reactions. Variables were not normally distributed and were analyzed with a Univariate GLM, which showed that there were significant differences between the cell types. OCY showed a significantly higher expression of 5-HT_{2B} mRNA, compared with the expression in OB and PF (p < 0.05 Mann-Whitney U with Bonferroni correction).

As 5-HT_{2B} receptor expression has been described for several mammalian tissues, we also studied 5-HT_{2B} receptor mRNA expression in several tissues isolated from fetal chickens. Northern blot analysis of the mRNA showed a non-significant higher 5-HT_{2B} receptor/GAPDH expression ratio in bone (calvaria, long bone) compared with other tissues (Fig. 3). Similar results were obtained by RT-PCR (data not shown).

As yet no function is known for serotonin in bone. However, stimulation of proliferation is one of the well known actions of serotonin in non-neuronal tissues (43–48). Osteocytes, which represent the highest differentiation stage of the osteoblastic lineage, do not proliferate (49) and therefore cannot be used in a proliferation assay. Therefore, we investigated whether α-methyl-5-HT, an analog of 5-HT, which preferentially binds to 5-HT_{2} receptors (51), regulated proliferation and differentiation in PF and OB, respectively.

In isolated and cultured OB, a concentration range of 10^{-7}–10^{-8} M α-methyl-5-HT did not affect alkaline phosphatase activity, a generally used marker for osteoblast differentiation, nor could it reverse the decrease in activity, which occurred at longer culture times. Fig. 4 shows the results of a representative experiment. However, many other experiments in which we varied seeding concentration (10^5, 3.3 × 10^4, 1.1 × 10^4, and 3.3 × 10^3 cells per well), medium composition (2% chicken serum or 0.2% bovine serum albumin) or culture time (1–3 days), showed similar results. The agonist α-methyl-5-HT did however significantly stimulate proliferation of PF in a dose-dependent manner (p < 0.05 Mann-Whitney U test with Bonferroni correction), in the same concentration range used for the alkaline phosphatase activity studies (Fig. 5). Prostaglandin E2 (10^{-5} M), a local factor known to stimulate proliferation in bone cells (50), was used as a positive control. Similar proliferation-stimulating effects were found with the other, more 5-HT_{2B} receptor selective 5-HT agonist, BW723C86 (51, 52), in the concentration range of 10^{-5}–10^{-3} M (Fig. 6); however these results were not significant. At higher concentrations of BW723C86 the proliferation stimulation decreased (although not significantly) by mechanisms that are not resolved yet. The proliferation stimulation by α-methyl-5-HT could be reversed by the addition of similar or higher concentrations ketanserin, a 5-HT_{2A}/5-HT_{2C} receptor-selective inhibitor in mammalian tissues (53) (Fig. 7) (p < 0.05 Mann-Whitney U test with Bonferroni correction), and by SB206553, a 5-HT_{2B}/5-HT_{2C} receptor-selective inhibitor (54) (Fig. 8) (p < 0.05 Mann-Whitney U test with Bonferroni correction). Particularly effective, appeared to be SB215505, a 5-HT_{2B} receptor-selective inhibitor (55) that significantly inhibited the α-methyl-5-HT-mediated proliferation stimulation at a 10-fold lower concentration (Fig. 9) (p < 0.05 Mann-Whitney U test with Bonferroni correction).

To ensure that the 5-HT_{2B} receptor mRNA expression is not unique for fetal or avian bone cells, we have determined mRNA expression of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors by RT-PCR in murine osteoblasts, isolated from adult mice. Both 5-HT_{2A} and 5-HT_{2B} receptor mRNAs were found to be (approximately equally) present, the expression of 5-HT_{2C} receptor was much lower (Fig. 10). As these cells represent highly differen-
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We examined the presence of 5-HT<sub>2B</sub> receptors in bone and bone cells. RNA was isolated from intestine, skin, brain, liver, heart, muscle, lung, tibiotarsus, calvaria (without periosteum), and periosteum obtained from 18-day-old fetal chickens, run on 1% agarose gels and transferred to nylon membranes. Northern blots were sequenentially hybridized with radioactive 5-HT<sub>2B</sub> receptor and GAPDH probes. Autoradiographs of the blots were obtained by exposure to Fuji Medical x-ray films at −80 °C. A, the results of a representative experiment. B, Northern blots were exposed to a PhosphorImager screen, scanned the next day, and net intensities were determined using ImageQuant software. Results are given as mean ± S.E.; n = 5.

PFP. The presence of α-methyl-5-HT inhibited the release of NO, both in stationary culture and during PFF.

DISCUSSION

In this paper we report the presence of 5-HT<sub>2B</sub> receptors in fetal chicken bone tissue and isolated bone cells. To our knowledge, this is the first report that suggests a direct role for serotonin and its receptors in bone metabolism. The receptor has been reported to be widely distributed in human and murine tissues (23, 24, 25, 29), which correlates well with our findings in chicken. In the present study, 5-HT<sub>2B</sub> receptor mRNA was found to be expressed in all tissues studied. Surprisingly, however, 5-HT<sub>2B</sub> receptor mRNA expression appeared to be the most pronounced in skeletal tissues. The 5-HT<sub>2B</sub> receptor mRNA expression in the various tissues may be due in part by the presence of receptor mRNA in endothelial tissue (51, 57, 58), which could be present in these organs. It is, however, very unlikely that the relatively high expression of 5-HT<sub>2B</sub> receptor mRNA in bone and bone cells is caused by contamination with endothelial cells. This is particularly evident for the highly purified OCY population, which has the highest 5-HT<sub>2B</sub> receptor mRNA expression. In addition, the murine osteoblast cultures, which also showed significant expression of 5-HT<sub>2B</sub> receptor mRNA, were tested and found to be negative for the presence of endothelial cells (56).
Roughly three major areas of serotonin activity have been described in the literature. The most investigated aspect of serotonin is its role as a neurotransmitter, which has a multitude of activities in the central nervous system (CNS). The diversity of the actions of serotonin is related to the existence of a large number of receptor subtypes (22), most of which have been found in the CNS. Interestingly, the 5-HT2B receptor has a low expression in the CNS compared with other receptor subtypes (26, 52).

A second important role of serotonin is its function in the regulation of the vascular tone. Serotonin may cause both vasoconstriction and vasodilatation. Vasoconstriction is caused by activation of 5-HT1D and 5-HT2A receptors on vascular smooth muscle cells (57), whereas vasodilatation has been attributed to activation of 5-HT2B receptors on endothelial cells, mediating the release of relaxing factors (51, 57, 58). Particularly these actions of serotonin are of interest for our finding that 5-HT2B receptors are present in such relatively high (in terms of mRNA that is) density in osteocytes. In some ways the osteocyte syncytium resembles the vascular system. The vascular tone is, apart from serotonin, also modulated by blood pressure and blood flow. High blood pressure and flow-induced shear stress stimulate the release of substances by the endothelial cells such as NO, which subsequently causes the vascular smooth muscle cells to relax, resulting in a decreased pressure and flow (59, 60). In bone, mechanical loading causes flow of interstitial fluid through the lacuno-canalicular system, resulting in shear stresses on the osteocyte syncytium that may be of the same order of magnitude as those encountered in the

FIG. 7. Ketanserin inhibits α-methyl-5-HT stimulated PF proliferation. PF were cultured under low serum conditions (0.1%) for 2 days and then postcultured for 24 h in the presence of α-methyl-5-HT (10^{-6} M) together with various concentrations of ketanserin. Two hours before culture was stopped, 3 × 10^{-5} M BrdUrd was added to the cultures. After fixation and immunostaining, BrdUrd-positive and -negative nuclei were counted. Proliferation is expressed as the percentage of positive nuclei. Results are given as mean ± S.E.; n ≥ 16. Asterisk, significant inhibition of α-methyl-5-HT-induced PF proliferation; p < 0.05 (Mann-Whitney U test with Bonferroni correction).

FIG. 8. SB206553 inhibits α-methyl-5-HT-stimulated PF proliferation. PF were cultured under low serum conditions (0.1%) for 2 days and then postcultured for 24 h in the presence of α-methyl-5-HT (10^{-6} M) together with various concentrations of SB206553. Two hours before the culture was stopped, 3 × 10^{-5} M BrdUrd was added to the cultures. After fixation and immunostaining, BrdUrd-positive and -negative nuclei were counted. Proliferation is expressed as the percentage of positive nuclei. Results are given as mean ± S.E.; n ≥ 21. Asterisk, significant inhibition of α-methyl-5-HT-stimulated PF proliferation; p < 0.05 (Mann-Whitney U test with Bonferroni correction).

FIG. 9. SB215505, a 5-HT_{2B} receptor selective inhibitor, inhibits α-methyl-5-HT-stimulated PF proliferation. PF were cultured under low serum conditions (0.1%) for 2 days and then postcultured for 24 h in the presence of α-methyl-5-HT (10^{-6} M) together with various concentrations of SB215505. Two hours before the culture was stopped, 3 × 10^{-5} M BrdUrd was added to the cultures. After fixation and immunostaining, BrdUrd-positive and -negative nuclei were counted. Proliferation is expressed as the percentage of positive nuclei. Results are given as mean ± S.E.; n = 40. Asterisk, significant inhibition of α-methyl-5-HT-induced PF proliferation; p < 0.05 (Mann-Whitney U test with Bonferroni correction).

FIG. 10. PCR analysis of 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor mRNAs in cultured mouse OB. Total RNA was extracted from cultured mouse OB and reverse transcribed. PCR reactions for three 5-HT_{2} receptor mRNAs were performed under the same conditions using two cDNA dilutions. A, the electrophoresis result of a representative experiment. The gel was photographed with a KODAK digital camera. M, 100-bp DNA Ladder (MBI Fermentas). B, results are given as mean ± S.E. of the fluorescence intensities analyzed with image analysis software at 1:30 dilution; n = 3.
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5-HT<sub>2B</sub> receptor in chicken bone cells, the fact that both the 5-HT<sub>2A</sub> receptor and the 5-HT<sub>2B</sub> receptor are present in mouse bone cells suggests that the 5-HT<sub>2A</sub> receptor may also be expressed in chicken bone cells. This could not be established as the nucleotide sequence of the chicken 5-HT receptors remain unknown so far, except for the partial sequence of the 5-HT<sub>2B</sub> receptor presented in this study. The apparent stimulation of PF proliferation by BW245C86, a selective agonist of the 5-HT<sub>2B</sub> receptor (52), together with the inhibition of PF proliferation by SB206553, a selective 5-HT<sub>2B/2C</sub> Receptor antagonist (54) and SB215505, a selective 5-HT<sub>2A</sub> receptor antagonist (55), strongly indicates that the 5-HT<sub>2B</sub> receptor is involved in the proliferative response to α-methyl-5-HT. The fact that ketanserin, a selective 5-HT<sub>2A/2C</sub> receptor antagonist (53), also inhibited the α-methyl-5-HT stimulation may signify that the inhibitor is not absolutely specific, or that the degree of its specificity depends on the animal species (23, 67, 68, 69), or that both receptors are involved.

What is the physiological role of PF proliferation stimulation by serotonin via the 5-HT<sub>2B</sub> receptor? Serotonin is released by coagulated platelets, the major site of serotonin storage in the body (70). One of the possible roles of serotonin in bone could therefore be the stimulation of osteoblast progenitor proliferation in bone fracture repair in conjunction with other local factors released at the site of fracture. In addition, a very exciting possibility is that serotonin is involved in the coupling of resorption and new bone formation in the remodeling of bone (71). Chopra and Anastasiades (72) have shown that bone sialoprotein (BSP) can be isolated using affinity chromatography on an immobilized serotonin column. Inversely, this may mean that in vivo serotonin is bound and concentrated by BSP in bone. During bone resorption, the bone-bound serotonin will be liberated and able to stimulate nearby osteoblast precursor cells, which will in turn differentiate into osteoblasts needed to restore the resorption cavity with new bone.

In conclusion, here we present the first report of the presence of serotonin receptors, in particular 5-HT<sub>2B</sub> Receptors, in bone. The receptors were shown to be functionally active in periosteal fibroblasts, by demonstrating the effects of specific agonists and antagonists on cell proliferation. The highest degree of 5-HT<sub>2B</sub> receptor expression in bone was found in osteocytes. The primary function of these cells is that of mechanosensors in the process of bone adaptation to loading. Serotonin was found to modulate the response of osteoblasts, the direct precursors of osteocytes, to pulsating fluid flow. We suggest that the 5-HT<sub>2B</sub> receptor and possibly other serotonin receptors play a role in the modulation of the mechanosensory function of osteocytes. In addition, serotonin could be involved in bone remodeling and fracture repair by stimulating the proliferation of osteoblast precursor cells.

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