Possible expression of a particular gamma-aminobutyric acid transporter isoform responsive to upregulation by hyperosmolarity in rat calvarial osteoblasts

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Abstract

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the brain, but widely distributed in different peripheral organs. We have previously shown the functional expression of $GABA_B$ receptors required for GABAergic signal input by cultured rat calvarial osteoblasts. This study focused on the possible functional expression of the machinery required for GABAergic signal termination such as GABA transporters. In rat calvarial osteoblasts cultured for 7 days, [^{3}H]GABA accumulation was observed in a temperature-, sodium- and chloride-dependent manner, consisting of a single component with a K_m value of 789.6 ± 9.0 μM and a V_{max} value of 4.4 ± 0.1 nmol/min/mg protein, respectively. Both nipecotic and L-2,4-diaminobutyric acids significantly inhibited [^{3}H]GABA accumulation in a concentration-dependent manner. Constitutive expression was seen with mRNA for the betaine/GABA transporter-1 (BGT-1) and taurine transporter (TauT), while hyperosmotic cultivation led to significant increases in both [^{3}H]GABA accumulation and BGT-1 mRNA expression without affecting TauT mRNA expression. Highly immunoreactive cells were detected for the BGT-1 isoform at the surface of trabecular bone of neonatal rat tibias. Sustained exposure to GABA significantly inhibited alkaline phosphatase (ALP) activity, but not cellular viability, at concentrations above 0.1 mM in osteoblasts cultured for 3 to 28 days. Nipecotic acid not only decreased ALP activity alone, but also further decreased ALP activity in osteoblasts cultured in the presence of GABA. These results suggest that the BGT-1 isoform may be functionally expressed by rat calvarial osteoblasts to play a hitherto unidentified role in mechanisms underlying hyperosmotic regulation of osteoblastogenesis.

1. Introduction

In the mammalian central nervous system (CNS), γ-aminobutyric acid (GABA) is one of the most abundant inhibitory amino acid neurotransmitters. In GABAergic synapses, GABA is synthesized from L-glutamic acid by glutamic acid decarboxylase, followed by the condensation into synaptic vesicles by vesicular GABA transporter and subsequent exocytotic release upon stimulation in a Ca^{2+}-dependent manner for the

signal output. A transient increase in extracellular GABA would usually lead to the activation of ionotropic $GABA_A$ and/or metabotropic $GABA_B$ receptors expressed at membranes in synaptic clefts for the signal input. For the signal termination, moreover, excess extracellular GABA is promptly removed from synaptic clefts through the incorporation by different high-affinity GABA transporter (GAT) isoforms into adjacent cells including glia and neurons (Borden, 1996; McIntire et al., 1997; Soghomonian and Martin, 1998; Watanabe et al., 2002). These GABAergic machineries are found in a variety of peripheral tissues in addition to the CNS, whereas little attention has been paid to the possible role of GABA in bone so far.

In bone, there are some different types of cells such as osteoblasts, osteocytes, osteoclasts and chondrocytes, which all

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have distinct roles and individual characteristics during bone development. In bone remodeling, maintenance of constant bone mass and renewal of bone are performed by osteoblasts and osteoclasts throughout life. Osteoblasts are bone-forming cells that synthesize extracellular matrix, whereas osteoclasts are bone-resorbing cells that remove bone matrix. Osteoblasts are derived from primitive mesenchymal cells and further differentiate into bone-lining cells and osteocytes (Harada and Rodan, 2003), whereas osteoclasts are multinucleated cells derived from the fusion of mononuclear hematopoietic precursors (Boyle et al., 2003). Dysfunction of and imbalance between osteoblasts and osteoclasts would lead to bone metabolic diseases such as osteoporosis, or more rarely, osteopetrosis (Rodan, 1998). Many endogenous factors are known to influence the activities of these cells. For instance, endocrine or paracrine (autocrine) control by bone morphogenetic protein, fibroblast growth factor, insulin-like growth factor, interleukin and transforming growth factor-β are quite important for the osteoblastic maturation and differentiation. In addition to these factors, the excitatory neurotransmitter glutamate is shown to play a pivotal role as a signal mediator through the glutamatergic signaling machineries expressed by osteoblasts in bone in recent studies by independent laboratories including ours. Ionotropic N-methyl-D-aspartic acid (NMDA) receptors are expressed in human and rat osteoblasts and osteoclasts (Patton et al., 1998; Espinosa et al., 1999; Gu et al., 2002; Hinoi et al., 2003), in fact, whereas both non-NMDA receptors (Hinoi et al., 2002a) and metabotropic glutamate receptors (Hinoi et al., 2001) are also expressed in cultured rat calvarial osteoblasts. Other machineries such as glutamate transporters (Mason et al., 1997; Huggett et al., 2000) and vesicular glutamate transporter (Hinoi et al., 2002b) are found in osteoblasts as well as osteoclasts. Through NMDA receptors, glutamate induces an elevation of the intracellular free Ca^{2+} level in a manner sensitive to an NMDA receptor antagonist in the human osteoblastic cell lines MG63 and SaOS-2 cells (Laketic-Ljubojevic et al., 1999).

Our previous study reveals that different NMDA receptor antagonists inhibit cellular differentiation and maturation in cultured calvarial osteoblasts through attenuation of the expression of runt-related transcription factor 2 (runx2) (Hinoi et al., 2003), a transcription factor essential for the regulation of osteoblast differentiation and bone formation (Ducy et al., 1997; Komori et al., 1999). Moreover, activation of particular metabotropic glutamate receptor subtypes, such as mGluR4 and mGluR8, regulates intracellular cAMP concentrations (Hinoi et al., 2001), with a concomitant reduction of NMDA-induced whole cell current in cultured osteoblasts (Gu and Publicover, 2000). On the basis of the aforementioned findings, glutamate is proposed to be one of the endogenous substances used for intercellular communications as a paracrine and/or autocrine signal mediator in bone. On a view related to the importance of the delicate balancing between excitatory glutamatergic signals and inhibitory GABAergic signals in a variety of neural functions, we have demonstrated the expression of mRNA for GABA$_B$ receptors, but not for GABA$_A$ receptors, in cultured rat calvarial osteoblasts on reverse transcription polymerase chain reaction (RT-PCR), with ability to inhibit developmental in-

creases in alkaline phosphatase (ALP) activity and Ca^{2+} accumulation, in addition to the formation of cAMP (Fujimori et al., 2002). In this study, therefore, we have examined the possible functional expression of GABA transporters required for the GABAergic signal termination in osteoblasts.

2. Materials and methods

2.1. Materials

A polyclonal antibody against rat betaine/GABA transporter-1 (BGT-1) was purchased from Chemicon International (Temecula, CA, USA). The anti-parathyroid hormone (PTH) receptor antibody was obtained from Biogenesis Ltd (Dorset, UK). Biotinylated anti-rabbit IgG antibody was provided by Vector Laboratories (Burlingame, CA, USA). γ-[2,3-^{3}H(N)]-Aminobutyric acid (1480 GBq/mmol) was obtained from Perkin-Elmer Life Science (Boston, MA, USA). GABA, choline chloride, sodium glucuronate and a C-TEST kit were obtained from Wako Pure Chemicals (Osaka, Japan). Taq polymerase was purchased from Takara (Tokyo, Japan). A Bio-Rad Protein Assay Kit was provided by Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals used were of the highest purity commercially available.

2.2. Preparation of primary cultured osteoblasts

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques. Osteoblasts were prepared from calvaria of 1- to 2-day-old Wistar neonatal rats by a sequential enzymatic digestion method as described previously (Hinoi et al., 2002a). In brief, rat calvaria were gently incubated at 37 °C for 10 min with 0.2% (w/v) collagenase in α-modified minimum essential medium (α-MEM), followed by the collection of cells in supernatants thus obtained. This incubation was consecutively repeated 5 times. Cells obtained during the last 3 digestion processes were altogether collected in α-MEM containing 10% (v/v) fetal bovine serum (FBS) and several antibiotics, followed by centrifugation at 250 g for 5 min. The pellets were suspended in α-MEM containing 10% FBS. Cells were plated at a density of 1×10^4 cells/cm^2 in appropriate dishes, and then cultured at 37 °C for different periods under 5% (v/v) CO$_2$ with medium change every 2 days. Throughout the experiments, α-MEM containing 10% FBS, 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate were used.

2.3. Determination of [^{3}H]GABA accumulation

Osteoblasts were cultured for 7 days, followed by washing with HEPES Krebs-Ringer (HKR) buffer (125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.25 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 10 mM HEPES and 10 mM D-glucose, pH 7.4) twice and subsequent further incubation in

HKR buffer at 37 °C for 1 h in 5% CO_2 incubator. Cells were then incubated with 1 μM [³H]GABA at 2 °C or 37 °C for 1 to 90 min, in either the presence or absence of different inhibitors, unless indicated otherwise (Takarada et al., 2004). Reaction was terminated by the careful aspiration of buffer, followed by rinsing with ice-cold HKR buffer containing 1 mM unlabeled GABA at 2 °C three times and subsequent solubilization of cells with 0.1 M NaOH for liquid scintillation spectrometry using 3 ml scintillation cocktail (clear sol I) (Hinoi et al., 2005). Nonspecific accumulation was defined in parallel experiments with 1 mM unlabeled GABA in the incubation mixture and subtracted from the total accumulation to calculate the specific uptake.

2.4. RT-PCR

Cultured osteoblasts were superficially washed with PBS twice, followed by the extraction of total RNA using ISOGEN (Nippon gene, Osaka, Japan) according to the manufacturer's instructions and subsequent synthesis of complementary DNA (cDNA) with 25 ng/μl oligo (dT)$_{18}$ primer, 0.5 mM deoxy nucleotide triphosphate mix and M-MLV Reverse Transcriptase. Reverse transcriptase reaction was run at 37 °C for 50 min, and an aliquot of synthesized cDNA was directly used for PCR performed in buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of deoxy nucleotide triphosphate, 20 pmol of each primer for the corresponding GAT isoforms and taurine transporter (TauT) (Table 1) and 2U of Taq DNA polymerase as reported elsewhere (Hinoi et al., 2005). Electrophoresis was run for an aliquot of PCR amplification products on a 2% (w/v) agarose gel, followed by the detection of DNA with ethidium bromide. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by ABI Prism 310 Genetic Analyzer (Perkin-Elmer) using a cycle sequencing kit. Quantitative analysis was done with the primer for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although results obtained on RT-PCR are by definition not quantitative, apparent semi-quantitative RT-PCR analysis was done at cycles below 30 using primers for GAPDH toward the comparative reference. PCR products were quantified by using a densitograph, followed by the calculation of ratios of expression of mRNA for each gene over that for GAPDH.

2.5. Immunohistochemistry

Immunohistochemical detection of the PTH receptor or the BGT-1 isoform was done as described previously with some

modifications (Hinoi et al., 2005). In brief, tibias were dissected from neonatal rats 1 day after birth, followed by the fixation with 4% (w/v) paraformaldehyde overnight and subsequent decalcification in 20% (w/v) EDTA for 2 days. Tibias were then immersed in 30% (w/v) sucrose overnight and sectioned at a thickness of 10 μm using a cryostat at −18 °C. Sections were further treated with 3% (v/v) hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity, followed by washing with 0.01 M phosphate-buffered saline (PBS). Sections were subjected to blocking with PBS containing 3% (v/v) normal goat serum at room temperature for 1 h and then with individual primary antibodies against PTH receptor, as a specific marker of osteoblasts, and BGT-1 subtype, which both were appropriately diluted in PBS containing 1% (v/v) normal goat serum and 0.2% (v/v) Triton X-100, at 4 °C overnight. After being washed in PBS, sections were reacted with a biotinylated anti-rabbit IgG antibody at room temperature for 2 h. After washing with PBS, sections were placed in the solution containing avidin and biotinylated peroxidase at room temperature for 1 h according to the manufacturer's instructions. After rinsing with PBS, sections were washed with 50 mM Tris–HCl buffer (pH 7.5) once, followed by the incubation for 10 to 15 min with 0.25 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl buffer (pH 7.5) containing 0.05% (v/v) hydrogen peroxide. After visualization, sections were dehydrated using conventional histological procedures, followed by mounting on cover slips using rapid mounting media.

2.6. Determination of ALP activity

Osteoblasts were plated at a density of 1×10^4 cells/cm² in 24-well dishes and cultured for different days. Cells were washed twice with cold PBS, and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% (v/v) Triton X-100. The assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM $MgCl_2$ and 10 mM *p*-nitrophenylphosphoric acid was added at a volume of 200 μl into 10 μl of cell suspensions, followed by the reaction for 30 min at 37 °C and subsequent immediate determination of the absorbance of *p*-nitrophenol at 405 nm. Protein concentration was determined with a Bio-Rad Protein Assay Kit, and ALP activity was standardized on the basis of both cellular protein content and incubation time for the representation as nmol/min/mg protein. The activity was linearly increased with incubation time up to 60 min under our experimental conditions (data not shown).

Table 1
Primers used

| Genes	Upstream (5′–3′)	Downstream (5′–3′)	Accession#	Estimated base pair
GAT-1	ACGCTTCGACTTCCTCATGTCCTGT	GAATCAGACAGCTTTCGGAAGTTGG	M59742	699 bp (296–994)
BGT-1	AGGGAGGCGTTCACCTCAGG	TTGGGCTCTGCAAGGCTGC	U28927	389 bp (2092–2480)
GAT-2	CCGGGTGTTCCGTAAGAAGAACCG	GTGTCGCTGGAAGAGTCAGCTCTGC	M95762	542 bp (1346–1887)
GAT-3	ACCCCAAGGCTGTCACTATG	TGTTGTACTTGAGCGGCTTG	M95763	480 bp (1142–1621)
TauT	CCAGGGAAGAGCCCAGGC	GGTCAGTCCACGGACCAGCAGCACC	M96601	735 bp (189–924)
GAPDH	GGTGAAGGTCGGTGTCAACGGATT	GATGCCAAAGTTGTCATGGATGACC	BC059110	502 bp (79–580)
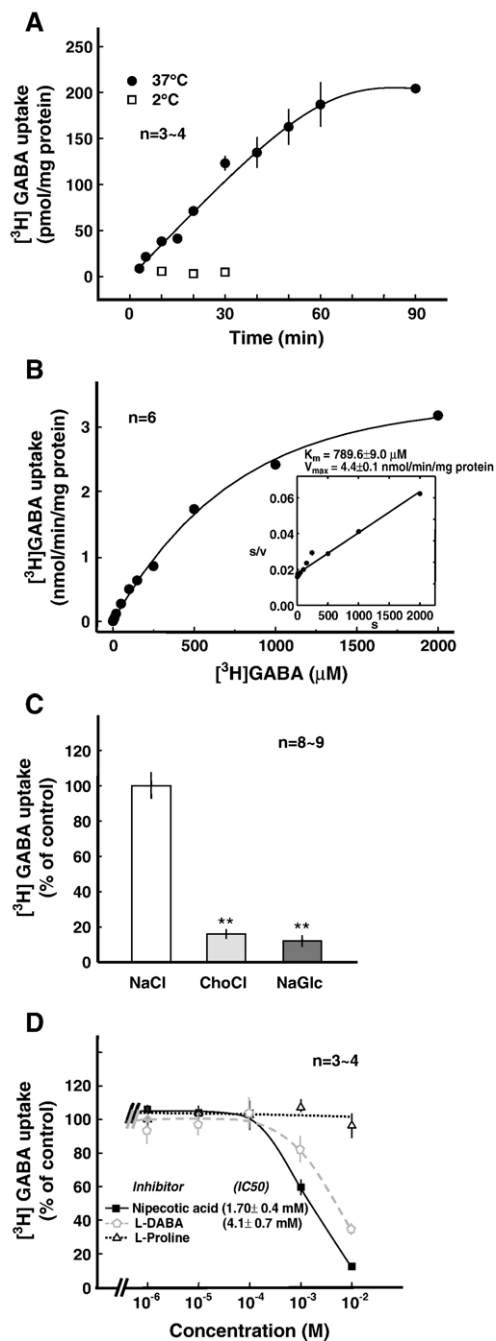
Fig. 1. [³H]GABA accumulation in cultured osteoblasts. (A) Osteoblasts cultured for 7 days were incubated with 1 μM [³H]GABA at 2 or 37 °C for different periods up to 90 min. (B) Osteoblasts were cultured for 7 days, followed by the incubation with 10 nM [³H]GABA in the presence of unlabeled GABA at different concentrations from 1 μM to 2 mM for 20 min at 37 °C. Hanes–Woolf plot analysis was done with the data obtained for [³H]GABA accumulation (inset). $V_{max}=4.4\pm0.1$ nmol/min/mg protein, $K_m=789.6\pm9.0$ μM. (C) Osteoblasts cultured for 7 days were incubated with 1 μM [³H] GABA at 37 °C for 20 min in HKR buffer where sodium chloride was replaced with equimolar choline chloride or sodium glucuronate as needed. (D) Osteoblasts cultured for 7 days were incubated with 1 μM [³H]GABA at 37 °C for 20 min in HKR buffer in either the presence of absence of different drugs at a concentration range of 1 μM to 10 mM. Values are the mean±S.E.M. obtained from different independent determinations shown as numbers in each figure. **$P<0.01$, significantly different from the control value obtained with normal HKR buffer. Abbreviations: ChoCl, choline chloride; NaGlc, sodium glucuronate.
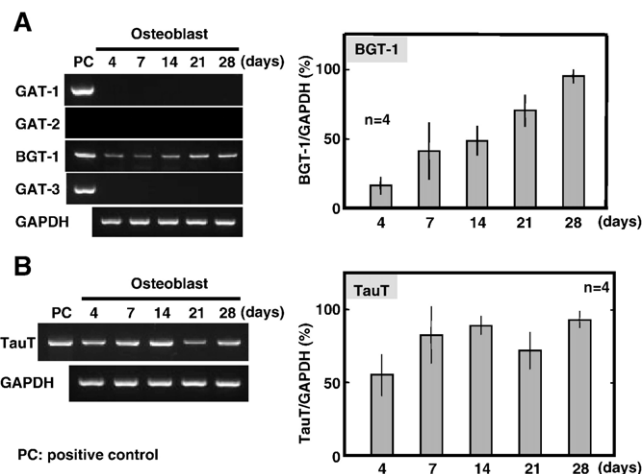

Fig. 2. Expression of particular GABA transporters in rat osteoblasts. Total RNA was extracted from osteoblasts cultured for 4 to 28 days, followed by semi-quantitative RT-PCR using specific primers for each GAT subtype (A) or TauT (B). Rat whole brain or liver was also subjected to RT-PCR as a positive control (PC). Values are the mean±S.E.M. in four separate determinations. **$P<0.01$, significantly different from the value obtained in osteoblasts cultured for 4 days.

2.7. Measurement of cell viability

Osteoblasts were cultured for 3 to 28 days in α-MEM containing 10% FBS, 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate in either the presence or absence of GABA at a concentration range of 100 μM to 1 mM. Osteoblasts cultured for different days were subjected to the determination of cellular viability based on mitochondrial succinic dehydrogenase activity using a cell counting kit-8 consisting of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay according to the manufacturer's instructions.

2.8. Data analysis

Results were expressed as the mean±S.E.M. and the statistical significance was determined by Students' t-test or one-way analysis of variance (ANOVA) with Scheffe's F posthoc test.

3. Results

3.1. [³H]GABA uptake in rat primary osteoblasts

In rat calvarial osteoblasts cultured for 7 to 28 days, marked expression was seen with both mRNA and corresponding protein for runx2 known to be a specific osteoblast marker and master regulator of osteoblast differentiation, while the marker protein of osteoblast maturation osteocalcin was exclusively expressed in cells cultured for 21 to 28 days (data not shown). In order to evaluate the possible expression of GABA transporters by osteoblasts, osteoblasts cultured for 7 days were incubated with 1 μM [³H]GABA in HKR buffer at 2 °C or 37 °C for different periods up to 90 min. [³H]GABA accumulation was almost linearly increased with incubation time up to 60 min and reached a plateau within 90 min at 37 °C, while no marked
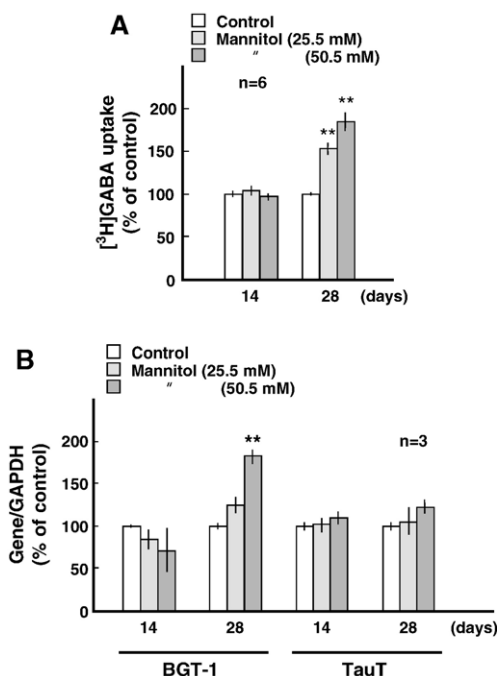
Fig. 3. Effect of hyperosmolarity on [^{3}H]GABA accumulation and mRNA expression. (A) Osteoblasts were cultured for 14 or 28 days in either the presence or absence of mannitol at a concentration range of 25.5 mM to 50.5 mM and harvested for the determination of [^{3}H]GABA accumulation. Osteoblasts were incubated with 1 μM [^{3}H]GABA at 37 °C for 20 min in HKR buffer. (B) Total RNA was extracted from osteoblasts cultured for 14 or 28 days in either the presence or absence of mannitol at 25.5 to 50.5 mM, followed by semi-quantitative RT-PCR using specific primers for BGT-1 or TauT. Values are the mean±S.E.M. of three to six separate determinations. **$P < 0.01$, significantly different from each control value obtained in osteoblasts cultured in the absence of mannitol.

accumulation of [^{3}H]GABA was seen for the initial periods of up to 30 min at 2 °C (Fig. 1A). Moreover, osteoblasts cultured for 7 days were incubated with [^{3}H]GABA at different concentrations of 1 μM to 2 mM at 37 °C for 20 min in HKR buffer for the determination of saturation isotherms. The accumulation of [^{3}H]GABA was increased in proportion to increasing concentrations of [^{3}H]GABA with saturation at a concentration over 2 mM (Fig. 1B). Hanes-Woolf plot analysis of these data revealed that [^{3}H]GABA accumulation consisted of a single component with a K_m value of 789.6±9.0 μM and a V_{max} value of 4.4±0.1 nmol/min/mg protein, respectively. Replacement of NaCl by equimolar choline chloride or sodium glucuronate led to almost complete abolition of the accumulation of [^{3}H]GABA for 20 min in osteoblasts cultured for 7 days (Fig. 1C). Osteoblasts cultured for 7 days were also incubated with 1 μM [^{3}H]GABA in HKR buffer containing different inhibitors of GABA transporters at a concentration range of 1 μM to 10 mM for 20 min at 37 °C. Of the 3 different inhibitors tested, the non-selective inhibitor nipecotic acid (IC$_{50}$=1.7±0.4 mM) was more potent in inhibiting [^{3}H]GABA accumulation than the neuronal GAT-1 inhibitor L-2,4-diaminobutyric acid (L-DABA, IC$_{50}$=4.1±0.7 mM), whereas L-proline did not significantly affect [^{3}H]GABA accumulation at concentrations used in osteoblasts cultured for 7 days (Fig. 1D).

3.2. Expression of GABA transporters in rat osteoblasts

An attempt was made to determine whether osteoblasts indeed express GABAergic signal termination machineries. As shown in Fig. 2A (left panel), constitutive mRNA expression was seen for BGT-1, but not for GAT-1, GAT-2 and GAT-3, isoforms of GABA transporters in rat calvarial osteoblasts cultured for 4 to 28 days. Sequencing analysis of amplified PCR products clearly confirmed the identity of mRNA for the BGT-1 isoform expressed in cultured osteoblasts, while semi-quantitative analysis reveled that BGT-1 mRNA was significantly increased in proportion to the duration of cultivation from 4 to 28 days (Fig. 2A, right panel). In addition to the BGT-1 isoform, furthermore, constitutive expression was seen with mRNA for the TauT capable of transporting GABA across plasma membranes in rat calvarial osteoblasts cultured for 4 to 28 days (Fig. 2B).

3.3. Effect of hyperosmolarity on [^{3}H]GABA uptake

As both the BGT-1 isoform and TauT are responsive to environmental osmotic changes, [^{3}H]GABA uptake was determined in osteoblasts cultured for 14 or 28 days in either the presence or absence of mannitol at 25.5 and 50.5 mM. Although no significant change was seen in [^{3}H]GABA accumulation in osteoblasts cultured under hyperosmotic conditions for 14 days irrespective of the concentration of mannitol added, while [^{3}H]GABA accumulation was almost doubled in osteoblasts cultured in the presence of 50.5 mM mannitol for 28 days compared to osteoblasts cultured under normal osmotic conditions (Fig. 3A, left panel). Mannitol at 25.5 mM was also effective in significantly increasing [^{3}H]GABA accumulation in osteoblasts cultured for 28 days without affecting that for 14 days.

On semi-quantitative RT-PCR analysis no significant change was found in BGT-1 mRNA expression in osteoblasts cultured in the presence of mannitol at 25.5 to 50.5 mM for 14 days,
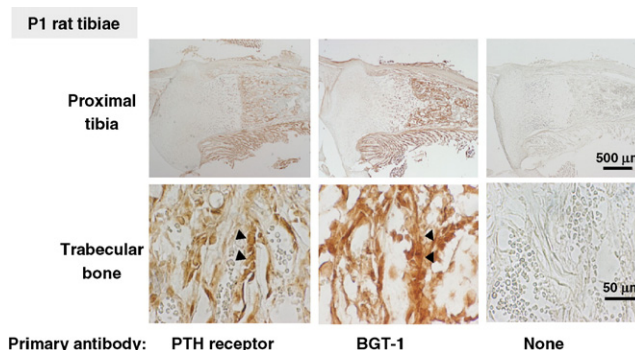

Fig. 4. Localization of BGT-1 isoform in neonatal rat tibia. Tibias were dissected from neonatal rats 1 day after birth, followed by the fixation with 4% (w/v) paraformaldehyde and subsequent dissection of frozen sections in a cryostat for immunochemical analysis using an antibody against the PTH receptor or the BGT-1 isoform. Negative control experiments were done without any primary antibody used. Black arrowheads indicate osteoblasts located on the surface of trabecular bone. Typical pictures are shown in the figures, while similar results were invariably obtained in at least three different animals.
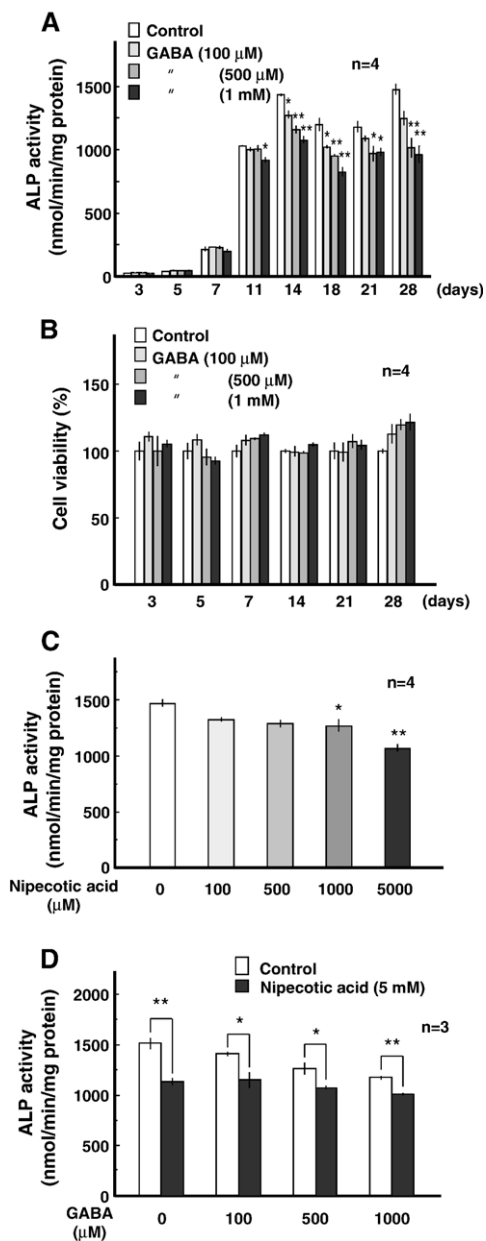
Fig. 5. Effects of GABA and nipecotic acid on bone formation in cultured rat osteoblasts. Osteoblasts were cultured in α-MEM in either the presence or absence of GABA at a concentration range of 100 μM to 1 mM for different periods from 3 to 28 days, followed by the determination of (A) the activity of ALP and (B) the number of living cells estimated by WST-8 assay. *$P<0.05$, **$P<0.01$, significantly different from each control value obtained in osteoblasts cultured in the absence of GABA. (C) Osteoblasts were cultured for 14 days in either the presence or absence of nipecotic acid at a concentration range of 100 μM to 5 mM and harvested for the determination of ALP activity. Values are the means±S.E.M. obtained in 4 separate determinations. *$P<0.05$, **$P<0.01$, significantly different from each control value obtained in osteoblasts cultured in the absence of nipecotic acid. (D) Osteoblasts were cultured for 14 days in either the presence or absence of 5 mM nipecotic acid in α-MEM containing GABA at a concentration range of up to 1 mM. Cells were then harvested for the determination of ALP activity. Values are the mean±S.E.M. obtained in three to four separate determinations.

while mRNA expression of the BGT-1 isoform was significantly increased in osteoblasts cultured for 28 days in the presence of mannitol at 50.5 mM (Fig. 3B, right panel). By

contrast, hyperosmolarity did not significantly affect the expression of TauT mRNA in osteoblasts cultured for 14 to 28 days irrespective of the concentration of mannitol used.

3.4. Expression of BGT-1 isoform in osteoblasts

Immunohistochemical analysis clearly showed high immunoreactivity for the BGT-1 isoform in osteoblasts located on rat trabecular bone of 1-day-old rat tibia (Fig. 4B, middle panels), where immunoreactive PTH receptors were highly condensed (Fig. 4B, left panels). However, no marked immunoreactivity was detected in any cells located in trabecular bone on analysis using sections not treated with the primary antibody (Fig. 4B, right panels).

3.5. Effects of GABA and nipecotic acid on osteoblastogenesis

The ALP activity was increased in proportion to culture periods from 3 to 28 days with a plateau within 14 days in osteoblasts (Fig. 5A). Sustained exposure to GABA significantly suppressed ALP activity in a concentration-dependent manner at concentrations of 100 μM to 1 mM when determined in osteoblasts cultured for 11 to 28 days, whereas no significant changes were seen in ALP activity at an early phase of up to 7 days even in the presence of GABA at different concentrations. However, GABA was ineffective in significantly altering the number of living cells at 100 μM to 1 mM in cultured osteoblasts irrespective of the duration of exposure (Fig. 5B). Therefore, sustained exposure to GABA would inhibit the cellular differentiation, but not proliferation, in cultured rat calvarial osteoblasts.

In order to evaluate the possible involvement of a GABA transporter in osteoblastic maturation, osteoblasts were cultured for 14 days in either the presence or absence of the non-selective GABA transport inhibitor nipecotic acid at a concentration range of 100 μM to 5 mM. As shown in Fig. 5C, nipecotic acid alone significantly decreased the activity of ALP in a concentration-dependent manner at concentrations tested in osteoblasts cultured for 14 days. To confirm the inhibition by GABA of maturation of osteoblasts, nipecotic acid at 5 mM was additionally exposed to osteoblasts cultured in the presence of GABA at different concentrations of up to 1 mM for 14 days. Sustained exposure to GABA resulted in a significant decrease in ALP activity at concentrations used in cultured osteoblasts, while the further addition of 5 mM nipecotic acid was more effective in significantly inhibiting ALP activity than that seen in the presence of GABA alone independent of the concentrations of GABA used (Fig. 5D).

4. Discussion

The essential importance of the findings obtained in this study is that rat osteoblasts expressed mRNA and corresponding protein for the particular GABA transporter isoform BGT-1 required for GABAergic signal termination in the CNS. In addition to these findings on mRNA expression and strong immunoreactivity for the BGT-1 isoform, [³H]GABA was indeed accumulated in a temperature-, sodium- and chloride-

dependent manner in primary cultured rat calvarial osteoblasts. To our knowledge, this is the first direct demonstration of the expression of both mRNA and corresponding protein for the BGT-1 isoform by osteoblasts with the activity of [³H]GABA accumulation. The data from kinetic evaluations, however, are not entirely similar to those seen in previous reports about the BGT-1 isoform in Madin-Darby canine kidney cells $K_m = 120 \mu M$) (Yamauchi et al., 1992) and LM (tk⁻) cells stably expressing human BGT-1 (GABA; $IC_{50} = 36 \mu M$) (Borden et al., 1995). The paradox could be accounted for by taking into consideration the possible expression of tissue-specific isoforms of BGT-1. There are several BGT-1 isoforms distinguishable by transcription start sites at different 5′-end exons (Takenaka et al., 1995). Although these isoforms are identical to each other at amino acid coding regions, a liver-specific isoform of BGT-1 containing an extended open reading frame apart from previous isoforms is identified (Burnham et al., 1996). The hepatic isoforms have additional 14 amino acids compared to the BGT-1 isoform expressed in canine (Yamauchi et al., 1992; Takenaka et al., 1995), murine (Lopez-Corcuera et al., 1992) and human (Borden et al., 1995) brains. The liver-specific isoform shows the activity of [³H]GABA accumulation with a K_m value of 0.3 mM in Xenopus oocytes injected with mRNA (Burnham et al., 1996) as seen in the present experimentation. These previous findings give rise to an assumption on the possible expression of un-identified BGT-1 isoforms specific for osteoblasts. As we have only confirmed the sequence of PCR products amplified using a BGT-1 specific primer, further clarification of the cDNA coding region about GABA transport molecules remains to be elu-cidated before drawing any conclusion.

It is noteworthy that constitutive expression of TauT mRNA was for the first time demonstrated in cultured rat calvarial osteoblasts irrespective of the cellular maturity. The fact that expression of BGT-1 mRNA, but not TauT mRNA, was signi-ficantly increased in response to hyperosmolarity together with the increased activity of [³H]GABA accumulation, however, argues in favor of an idea that osmolarity-sensitive [³H]GABA accumulation would be at least in part mediated by the BGT-1 isoform rather than the TauT in cultured calvarial osteoblasts. Although both BGT-1 isoform and TauT are shown to be responsive to hyperosmotic conditions (Yamauchi et al., 1992; Warskulat et al., 1997), present findings again emphasize the functional expression of the BGT-1 isoform responsive to hy-perosmolarity in rat osteoblasts. In contrast, mechanical loading is shown to downregulate mRNA expression in bone of the particular excitatory amino acid transporter (EAAT) isoform, glutamate aspartate transporter (GLAST), required for the CNS glutamatergic signal termination (Mason et al., 1997). Although no evidence is available for the responsiveness to hyperosmo-larity of any EAAT isoforms in the literature to date, one attractive but hitherto unproven speculation is that hyperosmo-larity could lead to downregulation of an EAAT isoform with concomitant upregulation of a GABA transporter for the deli-cate balancing between excitatory and inhibitory signal trans-duction in bone as seen in the brain. The fact that [³H]glutamate accumulation is drastically attenuated through a decrease in the V_{max} value during the period from 7 to 21 days in cultured

osteoblasts (Takarada et al., 2004) made us rather difficult to accurately and reproducibly assess [³H]glutamate accumulation in osteoblasts cultured under hyperosmotic conditions for 28 consecutive days as done with [³H]GABA accumulation.

We have shown the functional expression by osteoblasts of a particular GABA transporter required for the signal termination in the CNS, in agreement with our previous findings on the functional expression of $GABA_B$ receptors required for the signal input (Fujimori et al., 2002). Further analysis on other GABAergic signaling machineries is now under way in our laboratory. For example, constitutive mRNA expression was seen for the enzyme responsible for GABA synthesis from putrescine diamine oxidase, but not for either isoform of glutamate decarboxylase, in rat calvarial osteoblasts cultured for 7 to 28 days (unpublished data). The final conclusion should undoubtedly await the further analysis on the formation of GABA from the radiolabeled precursor putrescine in osteoblasts in vitro and in vivo. In addition, the possibility that GABA could be provided by cells adjacent to osteoblasts for the signal output and/or by blood circulation is not ruled out so far. In fact, the systemic administration of [¹⁴C]GABA leads to moderately selective localization of the radioactivity to hyaline cartilage in joint and vertebral column, in addition to costal and tracheal cartilages, in mice (Kuroda et al., 2000). As for vesicular GABA transporter responsible for GABA condensation into synaptic vesicles for subsequent exocytotic release in the CNS, our RT-PCR analysis revealed that mRNA was not expressed at a detectable level in osteoblasts cultured for 7 to 21 days (unpub-lished data). In contrast to the demonstration of exocytotic release of endogenous glutamate in cultured rat osteoblasts (Hinoi et al., 2002b) and chondrocytes (Wang et al., 2005), therefore, the absence of vesicular GABA transporter mRNA is not suggestive of the exocytotic release of GABA from osteo-blasts. There is a possibility that particular neurotransmitter transporters may be operative to a reverse direction in a Ca^{2+}-independent manner (Attwell et al., 1993; Levi and Raiteri, 1993), while a GABA transporter could mediate GABA efflux to maintain synaptic GABA concentrations in the brain (Phillis et al., 1994). Therefore, it is possible to speculate that the BGT-1 isoform may play a role in mechanisms underlying the export of intracellular GABA as an endogenous autocrine signal mediator for subsequent activation of $GABA_B$ receptors expressed by this bone-forming cell (Fujimori et al., 2002).

From this point of view, it should be noted that sustained exposure to GABA led to marked inhibition of the ALP activity in primary cultured rat calvarial osteoblasts when determined after the stage of cellular differentiation. The lack of effect of GABA on increases in the cell number throughout cell growth gives support to the proposal that GABA may inhibit differ-entiation rather than proliferation in osteoblasts. Although further analysis should be done with the effect of GABA on several markers other than ALP for osteoblastic maturation, extracellular GABA could also modulate the bone maturation determined by Ca^{2+} accumulation as shown with the $GABA_B$ receptor agonist baclofen in our previous study (Fujimori et al., 2002). The additive inhibition by a GABA uptake inhibitor with GABA makes it possible for us to speculate that extracellularly

accumulated GABA may play a role in mechanisms underlying the inhibition of cellular differentiation rather than proliferation in cultured rat calvarial osteoblasts. In fact, similarly potent inhibition is seen in both ALP activity and Ca^{2+} accumulation in osteoblastic MC3T3-E1 cells cultured in the presence of a $GABA_B$ receptors agonist (Fujimori et al., 2002). These findings suggest that extracellular GABA may inhibit osteoblastic differentiation through activation of $GABA_B$ receptors expressed by osteoblasts in bone. The exact mechanism as well as functional significance for the suppression by GABA of cellular differentiation, however, should be evaluated in future studies.

On the other hand, evidence is accumulating for the possible existence of the GABA synthetic pathway in hypertrophic chondrocytes in rat growth plate (Tamayama et al., 2001). A recent paper also shows the expression of mRNA and corresponding protein for both $GABA_A$ and $GABA_B$ receptors in rat growth plate chondrocytes as well as mouse chondrogenic cell line ATDC5 cells (Tamayama et al., 2005). Since the expression profile of GABA in rat growth plate is favorable for a wide-spectrum role of GABA in bone, GABA could act as a signal mediator not only in osteoblasts for the bone formation but also in chondrocytes for the articular joint formation. Accordingly, GABA could play a pivotal role in developmental skeletogenesis as seen with other endocrine, autocrine (paracrine) and/or neurocrine factors. Further investigation is of course required for the demonstration of GABAergic control mechanisms on bone remodeling as well as endochondral ossification. These include clarification of possible functional expression of GABAergic signaling machineries in osteoclasts and bone marrows. Evaluation of mechanisms relevant to the delicate balancing between excitatory glutamatergic and inhibitory GABAergic control would give us a clue to create a new therapeutic strategy for a variety of bone disorders associated with imbalance of bone metabolism between the formation by osteoblasts and the resorption by osteoclasts, such as osteoporosis and osteopetrosis, in addition to achondroplasia by chondrocytes.

It thus appears that the BGT-1 isoform could be functionally expressed with ability to modulate extracellular levels of GABA required for $GABA_B$ receptor activation through the active transportation across plasma membranes in cultured rat calvarial osteoblasts.

Acknowledgements

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