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Accumulation of [^{3}H]glutamate in cultured rat calvarial osteoblasts

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Abstract

In the present study, we have attempted to demonstrate constitutive and functional expression in bone of particular glutamate transporters (GluTs) required for signal termination in glutamatergic signaling process. Reverse transcription polymerase chain reaction revealed constitutive expression of mRNA for the neuronal GluT subtype excitatory amino acid carrier-1, in addition to glial subtypes such as glutamate aspartate transporter and glutamate transporter-1, in rat calvarial osteoblasts cultured for 7–21 days in vitro (DIV). The accumulation of [^{3}H]glutamate (Glu) occurred in a temperature- and sodium-dependent manner with pharmacological profiles similar to those for brain GluTs in osteoblasts cultured for 7 DIV, while three different agonists at ionotropic Glu receptors significantly inhibited the accumulation of [^{3}H]Glu in osteoblasts. Although [^{3}H]Glu accumulation consisted of a single component with a K_m value of 26.0 ± 5.8 μM and a V_{max} value of 960 ± 122 nmol/(min mg protein), respectively, in osteoblasts cultured for 7 DIV, in vitro maturation led to a significant decrease in V_{max} value to 290 ± 33 nmol/(min mg protein) without significantly affecting K_m values on 21 DIV. These results suggest that Glu could be incorporated into intracellular locations through glial and/or neuronal GluT subtypes expressed in cultured rat calvarial osteoblasts.

Keywords: Glutamate; GluTs; Osteoblasts; [^{3}H]Glutamate accumulation; EAAC1

1. Introduction

In agreement with our previous findings in adrenal [1] and pituitary [2] glands, recent molecular biological studies have raised the possibility that the central excitatory amino acid neurotransmitter L-Glu may be one of the endogenous factors used for intercellular communications as a paracrine and/or autocrine substance in bone [3]. RT-PCR analysis reveals constitutive expression of mRNA for certain subunits of *N*-methyl-D-aspartate (NMDA) [4–6], DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA) [7] and kainate (KA) [7] receptor subtypes of ionotropic Glu receptors (iGluRs), as well as for metabotropic Glu receptor (mGluR) subtypes [6], in primary cultures of rat osteoblasts. In patch-clamped cultured rabbit osteoclasts, moreover, an antagonist at the NMDA receptor channel inhibits whole-cell membrane currents induced by Glu, with concomitant prevention of cellular differentiation of osteoclasts cultured together with bone marrow and osteoblasts [8]. The NMDA receptor antagonist dizocilpine markedly diminishes increasing in both the activities of alkaline phosphatase and Ca^{2+} accumulation, as well as expression of osteocalcin, during cellular maturation through inhibition of expression of core binding factor α-1 in cultured rat calvarial osteoblasts [9].

In central glutamatergic synapses, both vesicular Glu transporters (VGLUTs) and Glu transporters (GluTs) need to be constitutively and functionally expressed for the condensation of Glu in synaptic vesicles and for the termination of signaling mediated by this amino acid, respectively, in addition to Glu receptors for the transformation of extracellular signals [10]. Vesicular Glu condensation is shown to involve both the brain-specific Na^+-dependent inorganic phosphate transporter (BNPI = VGLUT1) [10,11] and the differentiation-associated Na^+-dependent inorganic phosphate cotransporter

Abbreviations: DIV, days in vitro; EAAC1, excitatory amino acid carrier-1; EAAT, excitatory amino acid transporter; GLAST, glutamate aspartate transporter; GLT1, glutamate transporter-1; Glu, glutamate; GluT, glutamate transporter; RT-PCR, reverse transcription polymerase chain reaction; VGLUT, vesicular glutamate transporter

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(DNPI = VGLUT2) [12]. Expression of either VGLUT suffices for definition of a glutamatergic phenotype in endocrine cells [13] as shown in neurons [10–12]. An antagonist for AMPA receptors is shown to significantly inhibit the release of endogenous Glu in a concentration-dependent manner in MG-63 osteosarcoma cells [14]. Both RT-PCR and immunohistochemical analyses reveal constitutive expression of membrane and intracellular molecular machineries generally required for vesicular release of a central neurotransmitter in human MG-63, TE85, and SaOS-2 osteosarcoma cell lines as well as primary cultured rat and human osteoblasts [15]. Our recent study has clearly demonstrated exocytotic release of endogenous Glu in a Ca^{2+}-dependent manner through BNPI enriched in cultured osteoblasts [16].

GluTs are classified into five different subtypes including the GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 so far [17]. Of these GluT subtypes cloned to date, both the glial GLAST and GLT1 are shown to reside in bone for mRNA and corresponding proteins, with the neuronal EAAC1 being absent [18]. In GLAST knockout mice, however, no marked differences are seen in a variety of phenotypes compared with wild-type siblings [19]. These include mandible and long bone size, morphology, trabeculation, regions of muscle attachment, resorption lacunae, and areas of formation versus resorption of bone. On the basis of these findings, the authors claim the functionality of those Glu signaling machineries expressed in bone [19]. Possible compensatory activities by GluTs other than GLAST would be therefore conceivable. Nevertheless, little attention has been paid to studies on the possibility for osteoblasts to really accumulate Glu in a temperature- and sodium-dependent manner as seen for GluTs expressed in brain [17]. In the present article, therefore, we have attempted to demonstrate possible accumulation of [^{3}H]Glu as well as to confirm constitutive expression of particular GluT subtypes, including the neuronal EAAC1, in cultured rat calvarial osteoblasts.

2. Materials and methods

2.1. Materials

[^{3}H]L-Glu (1587.3 GBq/mmol) was purchased from Perkin-Elmer Life Sciences. QuickPrep Micro mRNA Purification Kit and Ready-To-Go You-Prime First-Strand Beads were supplied by Amersham Biosciences. Taq polymerase was obtained from Takara. Bio-Rad Protein Assay Kit was provided by Bio-Rad Laboratories. L-*Trans*-pyrrolidine-2,4-dicarboxylic acid (PDC), L-(−)-*threo*-3-hydroxyaspartic acid (THA) and (2S,1′S,2′R)-2-(carboxycyclopropyl)glycine (CCGIII) were purchased from Tocris. Other chemicals used were all of the highest purity commercially available.

2.2. Primary cultures

Osteoblasts were prepared from calvaria of 1-day-old Wistar rats by a sequential enzymatic digestion method previously reported [20] with minor modifications [6]. In brief, rat calvaria were gently incubated at 37 °C for 10 min with 0.1% collagenase and 0.25% trypsin in calcium and magnesium-free phosphate-buffered saline (PBS), followed by collection of cells in supernatants thus obtained. This incubation was repeated five times. Cells obtained during the last three digestions were pooled in α-modified minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and several antibiotics, followed by centrifugation at $250 \times g$ for 5 min. The pellets were suspended in α-MEM containing 10% FBS. Cells were plated with a density of 1×10^4 cm^{-2} to appropriate dishes, and then cultured at 37 °C for different periods under 5% CO_2. Medium was changed every 2 days. Throughout experiments, α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mM sodium β-glycerophosphate was used as mentioned previously [21].

Osteoblasts isolated here expressed the specific osteoblast marker and master regulator of osteoblast differentiation, polyomavirus enhancer binding protein 2αA/core binding factor-1 from 7 to 28 DIV, while the marker protein of osteoblast maturation osteocalcin was expressed in cells cultured for 21–28 DIV (data not shown). Alkaline phosphatase activity increased in proportion to duration of culture from 3 to 28 DIV in osteoblasts, while calcium was not detected in immature cultured cells up to 14 DIV but markedly increased with DIV from 14 to 28 days [7]. Under these culture conditions, osteoblastic cells were in a proliferative phase between 1 and 6 days with formation of several confluent cell layers, and subsequently by 10–11 days cells were more refractive and round than the background cell layers, followed by formation of some nodules and clusters with matrix. The matrix started to mineralize and calcify within 15 DIV (data not shown).

2.3. RT-PCR

Cultured osteoblasts were washed with PBS twice, followed by extraction of total mRNA using mRNA purification kit and subsequent synthesis of complementary DNA (cDNA) with 12.5 μM random hexamer primers and first-strand beads. The reverse transcriptase reaction was run at 37 °C for 60 min, and an aliquot of synthesized cDNA was directly used for PCR. PCR was 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 (dNTP), 20 pmol of each primer for the corresponding GluR subunits and two units of Taq DNA polymerase as described previously [22]. RT-PCR was performed with primers specific for each EAAT subtype. Cycling conditions used for 40 PCR cycle number were as follows:

GLAST, GLT1, EAAC1, EAAT4 and EAAT5, denaturation for 45 s at 95 °C, annealing for 40 s at 64 °C, extension for 40 s at 72 °C. Electrophoresis was run for an aliquot of PCR amplification products on a 2% agarose gel, followed by detection of DNA with ethidium bromide. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by AB1 Prism 310 Genetic Analyzer (Perkin-Elmer) using cycle sequencing kit.

2.4. Determination of [^{3}H]Glu accumulation

Osteoblasts were cultured for 7–21 DIV, followed by washing with HEPES Krebs Ringer (HKR) (125 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES and 10 mM D-glucose, pH 7.4) buffer twice and subsequent incubation in HKR buffer at 37 °C for 1 h in 5% CO_2 incubator. Cells were then incubated with 1 μM [^{3}H]Glu at 2 or 37 °C for 1–20 min unless indicated otherwise. Reaction was terminated by the aspiration of buffer, followed by rinsing with ice-cold HKR buffer containing 1 mM unlabeled Glu at 2 °C three times and subsequent solubilization with 0.1 M NaOH for liquid scintillation spectrometry using 3 mL scintillation cocktail (clear sol I). Protein concentration was determined with a Bio-Rad Protein Assay Kit.

2.5. Data analysis

Results are expressed as the mean ± S.E. and the statistical significance was determined by the Students' *t*-test or Scheffe's *F* post-hoc test after one-way analysis of variance.

3. Results

3.1. Expression of mRNA for GluTs

In order to analyze expression of mRNA for GluTs, total RNA was extracted from samples followed by purification of mRNA and subsequent RT-PCR. Rat whole brain exhibited constitutive expression of mRNA for GLAST, GLT1, EAAC1 and EAAT4, with expression of EAAT5 mRNA in rat retina (Fig. 1). In osteoblasts cultured for 7–21 DIV, mRNA was invariably expressed for GLAST, GLT1 and EAAC1, but not for either EAAT4 or EAAT5 irrespective of the maturity of cultured osteoblasts. Sequencing analysis on amplified PCR products clearly confirmed expression of mRNA for corresponding GluT subtypes in immature and mature cultured osteoblasts. In osteoblasts cultured for 7 or 21 DIV, however, no marked immunoblots were detected for either GLAST or GLT1 at the corresponding molecular weight positions on the gel

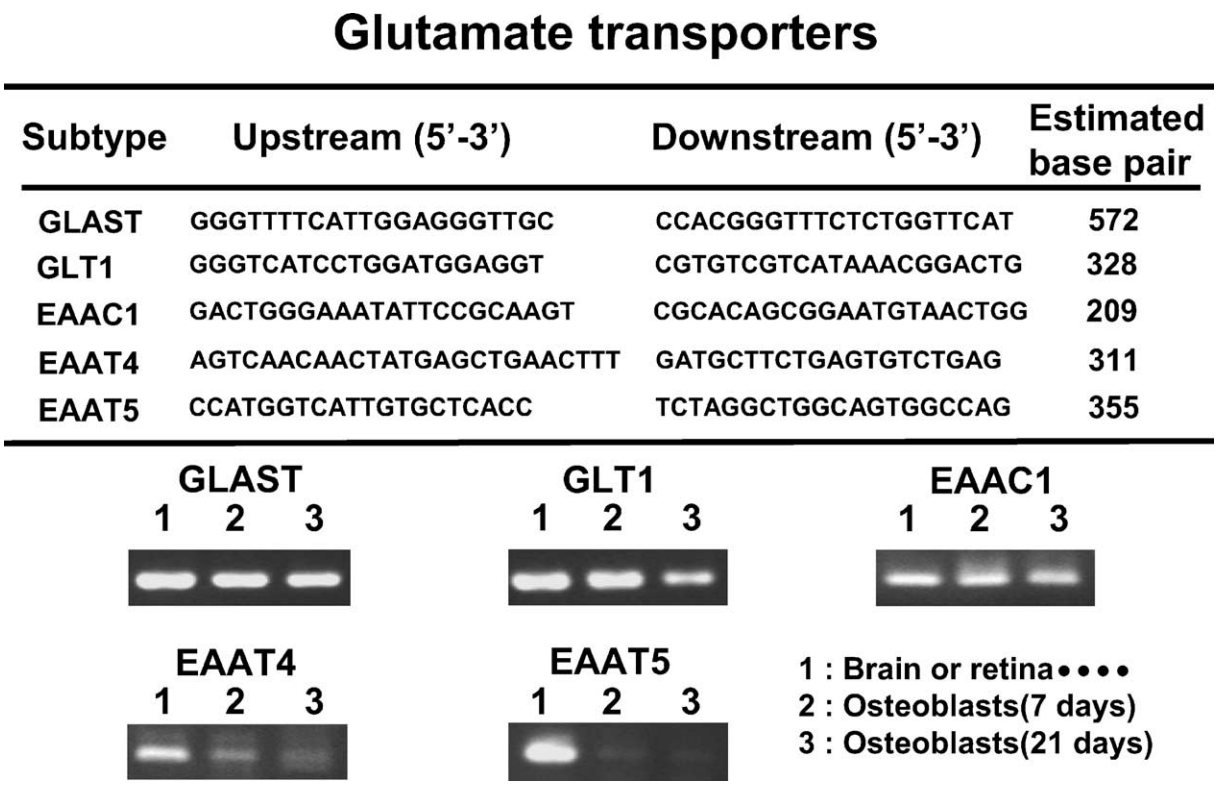

Fig. 1. Expression of mRNA for EAATs in cultured osteoblasts. Osteoblasts were cultured for 7 or 21 DIV, followed by isolation of mRNA and subsequent RT-PCR using primers specific for each EAAT. Rat whole brain or retina was also subjected to RT-PCR simultaneously done under the same experimental conditions. Typical pictures are shown in the figure with similar results in three separate determinations.

when determined by Western blotting assays under the experimental conditions used (data not shown).

3.2. [^{3}H]Glu uptake

Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu in HKR buffer at 2 or 37 °C for different periods up to 20 min, followed by rapid aspiration of buffer and subsequent rinsing with ice-cold buffer containing unlabeled Glu at 1 mM three times. Under these experimental conditions, [^{3}H]Glu accumulation was not markedly affected by the later addition of 1 mM unlabeled Glu in cultured osteoblasts (data not shown). The accumulation of [^{3}H]Glu almost linearly increased with incubation time up to 10 min and reached a plateau within 20 min at 37 °C, while no marked accumulation of [^{3}H]Glu was seen even 10 min after the initiation of incubation at 2 °C (Fig. 2a).

Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer with replacement of NaCl by choline chloride or sodium glucuronate at the same concentration. As shown in Fig. 2b, replacement of sodium chloride with choline chloride led to complete abolition of the accumulation of [^{3}H]Glu in osteoblasts. However, replacement of sodium chloride with sodium glucuronate did not significantly affect the accumulation of [^{3}H]Glu in osteoblasts cultured for 7 DIV.

3.3. Kinetics of [^{3}H]Glu uptake

Osteoblasts cultured for 7 or 21 DIV were incubated with different concentrations of [^{3}H]Glu at a concentration range of 1–250 μM at 37 °C for 5 min in HKR buffer for determination of saturation isotherms. The accumulation increased with increasing concentrations of [^{3}H]Glu followed by saturation at a concentration above 100 μM within a concentration range of up to 250 μM in osteoblasts cultured for 7 DIV (Fig. 3a). In osteoblasts cultured for 21 DIV, the accumulation of [^{3}H]Glu was much less than that found in osteoblasts cultured for 7 DIV at a substrate concentration range of below 250 μM.

Lineweaver–Burk plot analysis revealed that [^{3}H]Glu accumulation consisted of a single component within a substrate concentration range of 1–250 μM in osteoblasts cultured for 7 or 21 DIV (data not shown). Fig. 3b shows Woolf–Hanes plot analysis on [^{3}H]Glu accumulation in osteoblasts cultured for 7 DIV at a substrate concentration range of below 250 μM. In osteoblasts cultured for 7 DIV, [^{3}H]Glu accumulation was composed of one single component with a K_m value of 26.0 ± 5.8 μM and a V_{max} value of 960 ± 122 nmol/(min mg protein), respectively, at the substrate concentration range employed. In osteoblasts cultured for 21 DIV, by contrast, V_{max} value was significantly decreased for [^{3}H]Glu accumulation without a significant change in K_m value compared to those found in osteoblasts cultured for 7 DIV [V_{max} (pmol/(min mg protein)), 960 ± 122 versus 290 ± 33 ($P < 0.01$); K_m (μM), 26.0 ± 5.8 versus 42.1 ± 10.7].

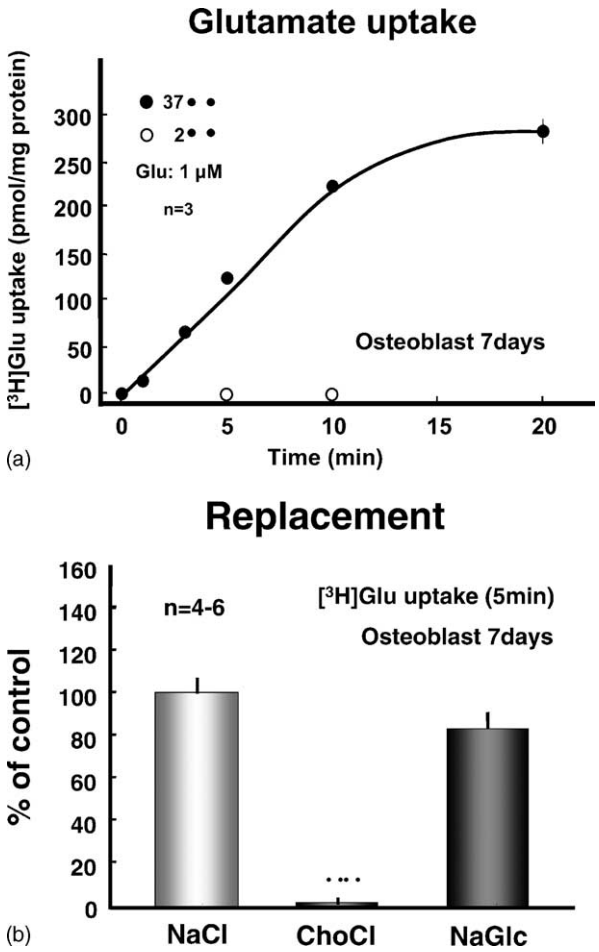

Fig. 2. [^{3}H]Glu accumulation in cultured osteoblasts. (a) Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu at 2 or 37 °C for different periods of up to 20 min in HKR buffer, followed by aspiration of buffer and subsequent rinsing with buffer containing unlabeled Glu at 1 mM. Values are the mean ± S.E. from three independent experiments. (b) Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer where sodium chloride was replaced with equimolar choline chloride or sodium glucuronate as needed. Values are the mean ± S.E. of 4–6 independent experiments. $^{**}P < 0.01$, significantly different from control value obtained with normal HKR buffer. Abbreviations: ChoCl, choline chloride; NaGlc, sodium glucuronate.

3.4. Pharmacology of [^{3}H]Glu uptake

Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu in HKR buffer containing different concentrations of several GluT inhibitors at a concentration range of 0.1 μM to 1 mM for 5 min at 37 °C. Of three different inhibitors of GluTs, L-THA (IC_{50} = 3.2 ± 0.97 μM) was most potent in inhibiting [^{3}H]Glu accumulation with progressively less potent inhibition by L-CCGIII (IC_{50} = 19.8 ± 1.9 μM) and L-*trans*-2,4-PDC (IC_{50} = 57.3 ± 3.0 μM) (Fig. 4a). The addition of AMPA at 1 mM alone did not significantly affect [^{3}H]Glu accumulation in osteoblasts cultured for 7 DIV, while the accumulation was

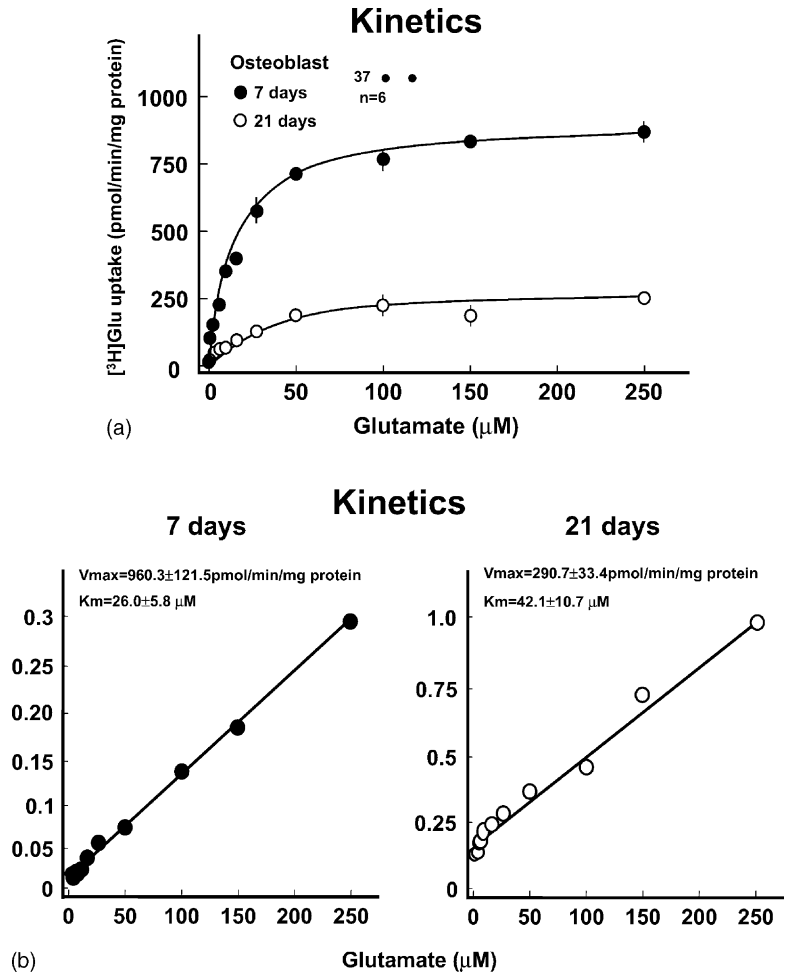

Fig. 3. Kinetic analysis on [^{3}H]Glu accumulation in cultured osteoblasts. (a) Osteoblasts were cultured for 7 or 21 DIV, followed by incubation with [^{3}H]Glu at different concentrations from 1 to 250 μM for 5 min at 37 °C for determination of [^{3}H]Glu accumulation. Values are the mean ± S.E. of six separate determinations. (b) Woolf–Hanes plot analysis was done with the data obtained for [^{3}H]Glu accumulation in osteoblasts cultured for 7 or 21 DIV.

significantly inhibited by AMPA in the presence of the inhibitor of AMPA receptor desensitization cyclothiazide at 50 μM (Fig. 4b). Similarly, [^{3}H]Glu accumulation was significantly inhibited by the addition of other iGluR agonists such as KA and NMDA at 1 mM.

Other compounds tested had no significant effects on [^{3}H]Glu accumulation in osteoblasts cultured for 7 DIV. These included ascorbic acid at 10–200 μg/mL, β-glycerophosphate at 1–20 mM, ouabain at 0.1–1 mM and valinomycin at 1–10 μM (data not shown). Neither GABA nor baclofen significantly affected [^{3}H]Glu accumulation at 1 mM in osteoblasts cultured for 7 DIV (data not shown).

4. Discussion

The essential importance of the present findings is that radiolabeled Glu was indeed transported in a temperature- and sodium-dependent manner in primary cultured rat calvarial osteoblasts. The accumulation of [^{3}H]Glu occurred with pharmacological profiles similar to those seen in neurons. To our knowledge, this paper deals with the first direct demonstration of the accumulation of [^{3}H]Glu in cultured rat calvarial osteoblasts. Several independent lines of evidence indicate the constitutive expression of both mRNA and corresponding proteins for the glial GluT subtypes GLAST and GLT1 in rat bone [18,23], which is also confirmed with mRNA on RT-PCR analysis using cultured rat calvarial osteoblasts in the present study. A previous immunohistochemical analysis reveals the constitutive expression of GLAST in both osteoblasts and osteocytes, and of GLT1 in mononuclear bone marrow cells, respectively [18]. In those previous studies, however, the temperature- and sodium-dependent accumulation of [^{3}H]Glu is not dealt with at all in osteoblastic or osteoclastic cells in contrast to the present investigation. The fact that osteoblasts were rinsed three times with buffer containing unlabeled Glu at a molar ratio of 1000 over radiolabeled Glu in the present experimentation, gives strong support for the idea that [^{3}H]Glu accumulation shown here is really derived from the net incorporation of the radiolabeled substrate into cultured cells but not to the simple adsorption and/or binding of the radioactive substrate to cellular surfaces.

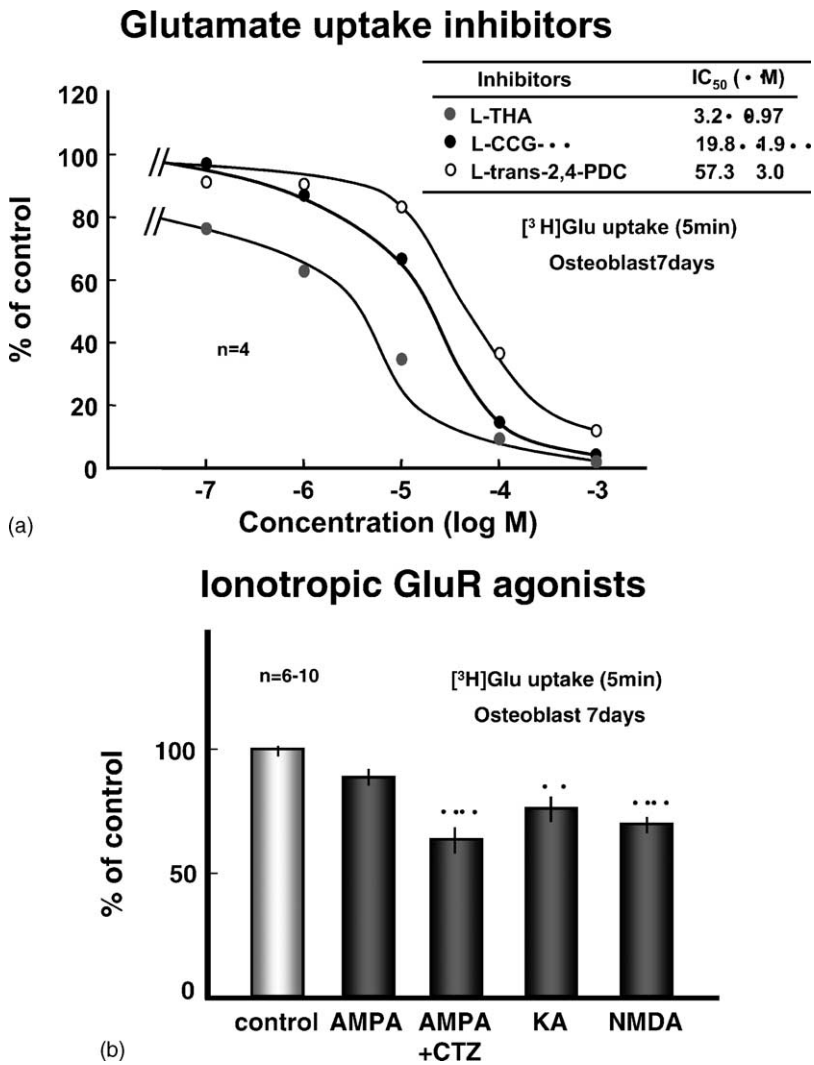

Fig. 4. Pharmacological analysis on [^{3}H]Glu accumulation in cultured osteoblasts. (a) Osteoblasts cultured for 7 DIV were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer containing different concentrations of the three different EAAT inhibitors at a concentration range of 0.1 μM to 1 mM. Values are the mean of four separate determinations. (b) Osteoblasts cultured for 7 DIV were incubated with [^{3}H]Glu at 37 °C for 5 min in HKR buffer containing three different iGluR agonists at 1 mM. Cyclothiazide (CTZ) at 50 was added simultaneously with 1 mM AMPA as needed. Values are the mean ± S.E. of 6–10 independent determinations. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in the absence of any agonists added or with normal HKR buffer.

The present study also has a credit on the exacerbation of [^{3}H]Glu accumulation during the course of cellular maturation in cultured rat calvarial osteoblasts. The functional significance as well as exact molecular mechanism for the maturation-dependent decrease in [^{3}H]Glu accumulation remains to be elucidated. Since our experimentation on Western blotting has failed to confirm the expression of GLAST detected in previous immunohistochemical analysis in rat osteoblasts and osteocytes [18] (unpublished observation), whether cellular maturation affects expression of particular subtypes of GluTs in cultured rat calvarial osteoblasts is not clear so far. Future studies on quantitative analysis of expression of mRNA and corresponding proteins for different GluT subtypes undoubtedly give us a clue for elucidation of molecular mechanisms underlying the maturation-dependent exacerbation of [^{3}H]Glu accumulation. Mechanical loading is shown to down-regulate GLAST expressed in osteocytes when determined on immunohistochemistry [18]. A view that immunohistochemistry is more sensitive than Western blotting to detect a small amount of corresponding proteins for GluT subtypes expressed in cultured rat calvarial osteoblasts is prevailing. *N*-Glycosylation is shown to have no impact on the transport activity of GLAST expressed in *Xenopus* oocytes [24], while a splice variant of GLAST is expressed in both bone and brain [23]. The possibility that the substrate [^{3}H]Glu would be unable to gain access to particular subtypes of GluTs expressed on cellular surfaces due to mineralization and calcification during maturation in cultured osteoblasts, however, is not ruled out so far.

It is noteworthy that the neuronal transporter EAAC1 was constitutively expressed, in addition to the glial transporters GLAST and GLT1, irrespective of cellular maturity in cultured osteoblasts. In recent studies, several novel

proteins are isolated and characterized with regard to the selective interaction with the neuronal GluT subtypes EAAC1 and EAAT4 through the respective intracellular carboxyl terminal domains in rat brains [25,26]. The individual interactions lead to a marked decrease in the activity of [^{3}H]Glu accumulation by EAAC1 [25], but a profound increase in that by EAAT4 [26]. These previous findings raise the possibility that hitherto unidentified proteins could be expressed for modulation of the activity of [^{3}H]Glu accumulation through the interaction with the glial GluT subtype GLAST and/or GLT1 during the cellular maturation in cultured rat calvarial osteoblasts. From this point of view, conventional determination of [^{3}H]Glu accumulation is still crucial for elucidation of the functional significance as well as exact mechanism for regulation of cellular homeostasis by different GluT subtypes expressed in particular neuronal and non-neuronal tissues, in addition to molecular biological strategies including RT-PCR, Western and Northern blotting, in situ hybridization and immunohistochemistry.

In our hands, particular subtypes of iGluRs [6,7] and mGluRs [6] are functionally expressed together with exocytotic and Ca^{2+}-dependent release through the VGLUT BNPI [16] in cultured rat calvarial osteoblasts, in addition to expression of mRNA for several subtypes of GluTs demonstrated here. Functional expression of these molecular machineries seems to be a prerequisite for the enrichment of Glu signaling system in osteoblasts, but does not completely fulfill the criteria as an autocrine/paracrine mediator. Although an alternative explanation that bone has glutamatergic innervations [27] is conceivable, the possible presence of glutamatergic neurons in cultured osteoblasts is inconceivable. Bone indeed has inputs from both sympathetic and sensory nerve fibers [28], while intracerebroventricular infusion of leptin leads to inhibition of bone formation through central neurons in leptin-deficient and wild-type mice [29]. Glutamatergic innervations would be distributed not only in the CNS, but also in peripheral non-neuronal tissues including bone. Glu could be classified into autacoids as done with histamine and serotonin, whereas a concept for the paracrine and/or autocrine signal mediator should be re-considered for establishment of the autocrine hypothesis for Glu. The lack of phenotypic alterations of the bone growth in mice knocked out of GLAST [19] would be brought about through the activity of Glu transport compensated by other EAAT subtypes co-expressed in osteoblasts even in the absence of GLAST.

The significant inhibition argues in favor of the previous proposition that particular subunits of three different iGluRs are constitutively and functionally expressed in cultured osteoblasts. In particular, the potentiation by cyclothiazide strongly suggests the expression of AMPA receptors known to undergo rapid desensitization [30] in cultured osteoblasts, which is in good agreement with our previous findings on mRNA expression [7] and exocytotic release [16]. The addition of one of these three iGluR agonists invariably facilitates the Ca^{2+}-dependent release of endogenous Glu in cultured rat calvarial osteoblasts [16]. This correlates well with the assumption that the inhibition could be due to facilitated release by these iGluR agonists of [^{3}H]Glu once incorporated into cultured osteoblasts during incubation. The medium α-MEM used here for cultivation contains approximately 500 μM Glu that is sufficient to saturate iGluRs and mGluRs judging from K_D values reported in the literature. Calvarial osteoblasts undergo severe apoptotic cell death following the exchange of culture medium from α-MEM to Dulbecco's modified Eagle medium not containing Glu, which is fully protected by the addition of pyruvate or cysteine [21]. Accordingly, GluTs are not only required for the termination of Glu signaling, but also needed for the disinhibition of the Glu/cystine antiporter x^{c-} [31], through reduction of extracellular Glu concentrations. The antiporter is essential for the biosynthesis of glutathione that is also effective in preventing the cell death by medium exchange described above (unpublished observation). Finally, Glu could at least in part participate in mechanisms underlying cellular proliferation and/or differentiation for maturation through an interaction with particular subtypes of iGluRs, mGluRs, VGLUT and GluTs constitutively expressed in osteoblasts.

It thus appears that extracellular Glu may be incorporated into intracellular locations through particular subtypes of GluTs for possible subsequent events such as signal termination and/or glutathione synthesis in osteoblasts. Search for inhibitors of osteoblastic GluTs could be of a great benefit for the elucidation of molecular mechanisms underlying crisis of a variety of bone diseases associated with malfunction of osteoblasts including osteoporosis.

Acknowledgments

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References

[1] Yoneda Y, Ogita K. Localization of [^{3}H]glutamate binding sites in rat adrenal medulla. Brain Res 1986;383:387–91.
[2] Yoneda Y, Ogita K. [^{3}H]Glutamate binding sites in the rat pituitary. Neurosci Res 1986;3:430–5.
[3] Skerry TM, Genever PG. Glutamate signalling in non-neuronal tissues. Trends Pharmacol Sci 2001;22:174–81.
[4] Chenu C, Serre CM, Raynal C, Burt-Pichat B, Delmas PD. Glutamate receptors are expressed by bone cells and are involved in bone resorption. Bone 1998;22:295–9.
[5] Laketic-Ljubojevic I, Suva LJ, Maathuis FJ, Sanders D, Skerry TM. Functional characterization of *N*-methyl-D-aspartic acid-gated channels in bone cells. Bone 1999;25:631–7.

[6] Hinoi E, Fujimori S, Nakamura Y, Yoneda Y. Group III metabotropic glutamate receptors in cultured rat calvarial osteoblasts. Biochem Biophys Res Commun 2001;281:341–6.
[7] Hinoi E, Fujimori S, Takemori A, Kurabayashi H, Nakamura Y, Yoneda Y. Demonstration of expression of mRNA for particular AMPA and kainate receptor subunits in immature and mature cultured rat calvarial osteoblasts. Brain Res 2002;943:112–6.
[8] Peet NM, Grabowski PS, Laketic-Ljubojevic I, Skerry TM. The glutamate receptor antagonist MK801 modulates bone resorption in vitro by a mechanism predominantly involving osteoclast differentiation, FASEB J 13:2179–85.
[9] Hinoi E, Fujimori S, Yoneda Y. Modulation of cellular differentiation by *N*-methyl-D-aspartate receptors in osteoblasts. FASEB J 2003;17: 1532–4.
[10] Bellocchio EE, Reimer RJ, Fremeau Jr RT, Edwards RH. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 2000;289:957–60.
[11] Takamori S, Rhee JS, Rosenmundm C, Jahr R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 2000;407:189–94.
[12] Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, et al. Molecular cloning of a novel brain-type Na^+-dependent inorganic phosphate cotransporter. J Neurochem 2000;74:2622–5.
[13] Hayashi M, Otsuka M, Morimoto R, Hirota S, Yatsushiro S, Takeda J, et al. Differentiation-associated Na^+-dependent inorganic phosphate cotransporter (DNPI) is a vesicular glutamate transporter in endocrine glutamatergic systems. J Biol Chem 2001;276:43400–6.
[14] Genever PG, Skerry TM. Regulation of spontaneous glutamate release activity in osteoblastic cells and its role in differentiation and survival: evidence for intrinsic glutamatergic signaling in bone. FASEB J 2001; 15:1586–8.
[15] Bhangu PS, Genever PG, Spencer GJ, Grewal TS, Skerry TM. Evidence for targeted vesicular glutamate exocytosis in osteoblasts. Bone 2001;29:16–23.
[16] Hinoi E, Fujimori S, Takarada T, Taniura HY, Yoneda Y. Facilitation of glutamate release by ionotropic glutamate receptors in osteoblasts. Biochem Biophys Res Commun 2002;297:452–8.
[17] Danbolt NC. Glutamate uptake. Prog Neurobiol 2001;65:1–105.
[18] Mason DJ, Suva LJ, Genever PG, Patton AJ, Steuckle S, Hillam RA, et al. Mechanically regulated expression of a neural glutamate transporter in bone: a role for excitatory amino acids as osteotropic agents? Bone 1997;20:199–205.
[19] Gray C, Marie H, Arora M, Tanaka K, Boyde A, Jones S, et al. Glutamate does not play a major role in controlling bone growth. J Bone Miner Res 2001;16:742–9.
[20] Collin P, Nefussi JR, Wetterwald A, Nicolas V, Boy-Lefevre ML, Fleisch H, et al. Expression of collagen, osteocalcin, and bone alkaline phosphatase in a mineralizing rat osteoblastic cell culture. Calcif Tissue Int 1992;50:175–83.
[21] Hinoi E, Fujimori S, Takemori A, Yoneda Y. Cell death by pyruvate deficiency in proliferative cultured calvarial osteoblasts. Biochem Biophys Res Commun 2002;294:1177–83.
[22] Hinoi E, Yoneda Y. Expression of GluR6/7 subunits of kainate receptors in rat adenohypophysis. Neurochem Int 2001;38:539–47.
[23] Huggett J, Vaughan-Thomas A, Mason D. The open reading frame of the Na^+-dependent glutamate transporter GLAST-1 is expressed in bone and a splice variant of this molecule is expressed in bone and brain. FEBS Lett 2000;485:13–8.
[24] Conradt M, Storck T, Stoffel W. Localization of *N*-glycosylation sites and functional role of the carbohydrate units of GLAST-1, a cloned rat brain L-glutamate/L-aspartate transporter. Eur J Biochem 1995;229: 682–7.
[25] Lin CI, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, et al. Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. Nature 2001;410:84–8.
[26] Jackson M, Song W, Liu MY, Jin L, Dykes-Hoberg M, Lin CI, et al. Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. Nature 2001;410:89–93.
[27] Serre CM, Farlay D, Delmas PD, Chenu C. Evidence for a dense and intimate innervation of the bone tissue, including glutamate-containing fibers. Bone 1999;25:623–9.
[28] Hohmann EL, Elde RP, Rysavy JA, Einzig S, Gebhard RL. Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing nerve fibers. Science 1986;232:868–71.
[29] Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 2000;100:197–207.
[30] Trussell LO, Zhang S, Raman LM. Desensitization of AMPA receptors upon multiquantal neurotransmitter release. Neuron 1993;10: 1185–96.
[31] Sato H, Tamba M, Okuno S, Sato K, Keino-Masu K, Masu M, et al. Distribution of cystine/glutamate exchange transporter, system x^{c-}, in the mouse brain. J Neurosci 2002;22:6028–33.