FEBS Letters 584 (2010) 817–824

FEBS Letters

journal homepage: www.FEBSLetters.org

Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts

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ARTICLE INFO

Article history:
Received 10 September 2009
Revised 11 December 2009
Accepted 5 January 2010
Available online 12 January 2010

Edited by Zhijie Chang

Keywords:
Acetylcholine
Cholinergic receptor
Cholinergic component
Osteoblast
Proliferation
Differentiation

ABSTRACT

Recent studies have indicated that acetylcholine (ACh) plays a vital role in various tissues, while the role of ACh in bone metabolism remains unclear. Here we demonstrated that ACh induced cell proliferation and reduced alkaline phosphatase (ALP) activity via nicotinic (nAChRs) and muscarinic acetylcholine receptors (mAChRs) in osteoblasts. We detected mRNA expression of several nAChRs and mAChRs. Furthermore, we showed that cholinergic components were up-regulated and subunits/subtypes of acetylcholine receptors altered during osteoblast differentiation. To our knowledge, this is the first report demonstrating that osteoblasts express specific acetylcholine receptors and cholinergic components and that ACh plays a possible role in regulating the proliferation and differentiation of osteoblasts.

1. Introduction

A growing body of literature has demonstrated that neuronal factors such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide, and substance P directly affect the differentiation of both osteoblasts and osteoclasts and that the sympathetic nervous system regulates bone remodeling [1–3].

Increasing evidence indicates that acetylcholine (ACh), a well-known neurotransmitter, is involved in the regulation of basic functions such as proliferation, differentiation, cell–cell contact, immune functions, secretion, and absorption in non-neuronal cells [4,5]. Immune cell function is regulated by its own cholinergic system. For example, muscarinic ACh receptors (mAChRs) play a crucial role in the cytokine production and the differentiation of T cells [6]. In lung, all components needed for an autocrine cholinergic signaling pathway are expressed in airway bronchial epithelial cells, and ACh acts as an autocrine growth factor in small cell lung carcinoma [7,8]. ACh attenuates macrophage activation and decreases the production of proinflammatory mediators by macrophages stimulated with endotoxin [9]. In rodent models of endotoxemia and hemorrhagic shock, stimulation of the efferent vagus nerve dampens macrophage activation [5]. In addition, many non-neuronal cells, including keratinocytes, lymphocytes, placental trophoblasts, embryonic stem cells, epithelial cells, and endothelial cells, can synthesize ACh and release autocrine or paracrine hormones [10–12]. Non-neuronal cells that possess cholinergic components uptake choline by means of the high affinity choline transporter (CHT1) and then synthesize ACh by choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (acetyl-CoA). ACh is translocated into small synaptic vesicles by vesicular ACh transporter (VAChT) and is released via exocytosis. Once released, ACh exerts its cellular functions via mAChRs, including 5 subtypes (M1, M2, M3, M4 and M5), and nicotinic ACh receptors (nAChRs), including 16 subunits (α1, α2, α3, α4, α5, α6, α7, α9,

Abbreviations: ACh, acetylcholine; Nic, nicotine; Mus, muscarine; CCh, carbachol; Atr, atropine; Mec, mecamylamine; nAChRs, nicotinic acetylcholine receptors; mAChRs, muscarinic acetylcholine receptors; VAChT, vesicular acetylcholine transporter; ChAT, choline acetyltransferase; CHT1, high affinity choline transporter; AChE, acetylcholinesterase; VIP, vasoactive intestinal peptide; BrdU, 5-bromo-2′-deoxyuridine; ALP, alkaline phosphatase; pOB, primary osteoblasts

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.01.001

α10, β1, β2, β3, β4, γ, δ and ε). Finally, ACh is rapidly degraded into choline and acetate by acetylcholinesterase (AChE) [12].

Periosteum, which consists of an outer layer of dense connective tissue with resident fibroblasts and an inner cellular layer containing immature osteoblasts and osteochondral progenitors, receives two classes of sympathetic innervation: noradrenergic fibers associated with the vasculature and non-adrenergic VAChT- and VIP-immunoreactive fibers associated with the parenchyma [13–15]. Asmus et al. have demonstrated that axons decrease their expression of catecholaminergic properties and acquire VAChT- and VIP-immunoreactivity on contact with the periosteum during development in vivo and after transplantation and that osteoblasts induce cholinergic function in cultured sympathetic neurons [16,17].

It is conceivable that VIP and ACh derived from VAChT- and VIP-immunoreactive fibers affect osteoblasts in the periosteum. Previous studies have demonstrated that osteoblasts express VIP receptors and that VIP stimulates the activity and mRNA expression of alkaline phosphatase (ALP) [18–20]. However, it remains unknown how ACh acts on osteoblasts and whether osteoblasts have cholinergic receptors and components. We hypothesized that ACh plays a functional role in bone metabolism. In the present study, we investigated the action of ACh during osteoblastic proliferation and differentiation and the mRNA expression of cholinergic receptors and components for ACh synthesis and release in osteoblasts.

2. Materials and methods

2.1. Reagents and antibodies

2-Acetoxy-*N*,*N*,*N*-trimethylethanaminium chloride (ACh), (−)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt (nicotine; Nic), (2*S*,4*R*,5*S*)-(4-hydroxy-5-methyl-tetrahydrofuran-2-ylmethyl)-trimethyl-ammonium chloride (muscarine, Mus), 2-carbamoyloxy-ethyl-trimethyl-azanium chloride (carbachol, CCh), (8-methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenylpropanoate (atropine, Atr), and *N*,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride (mecamylamine, Mec) were obtained from Sigma–Aldrich. Antibodies against Cdk4 (C-22), Cdk6 (DCS-83), cyclin D1 (HD11) and β-actin (C-4) were obtained from Santa Cruz Biotechnologies (California, USA).

2.2. Cell culture

Murine primary osteoblasts (pOB) were isolated from calvarie taken from newborn ddY mice (SLC, Shizuoka, Japan). The experiments were conducted according to the institutional ethical guidelines for animal experiments. Neonatal mouse calvaria were dissected free of adherent soft tissue, washed in PBS, and sequentially digested with 0.2% dispase and 0.1% collagenase. pOB and the mouse pre-osteoblastic cell line MC3T3-E1 were maintained in growth medium, consisting of α-modified minimum essential medium (α-MEM) (WAKO, Osaka, Japan) with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France). For osteoblastic differentiation assay, cells were cultured in growth medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) (conditioned medium). The growth medium and the conditioned medium were changed every three days. All cultures were maintained at 37 °C in humidified air including 5% CO_2 and were passaged every seven days.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

DNA synthesis was assessed with a Cell Proliferation Biotrack ELISA System (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's recommendations. This assay measures BrdU incorporation during DNA synthesis by proliferating cells. Briefly, cells were plated to a density of 5×10^3 cells/well in 96-well plates and allowed to proliferate for 24 h. Next, their growth was arrested by incubation for 24 h in serum-free medium. Cells were then treated with stimulants (ACh, CCh, Mus, and Nic) at the indicated doses in growth medium for 60 h. To assess the effects of inhibitors (Mec and Atr), growth-arrested cells were treated with growth medium for 60 h plus stimulants in the presence or absence of inhibitors at the indicated doses, which were added 1 h before treatment with stimulants. The absorbance was measured at 450 nm using a Model 680 XR plate reader (BIO RAD, California, USA). Measurements are represented by the means of at least three independent experiments, with each data point based on six replicates.

2.4. Reverse-transcriptase polymerase chain reaction (RT-PCR)

After reaching confluence, cells were incubated in conditioned medium in the presence or absence of ACh (1nM) for 14 days. Total RNA (2 μg) was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) and was subjected to RT-PCR using a SuperScript One-Step RT-PCR kit (Invitrogen, California, USA) according to manufacturer's instructions. The gene-specific primer pairs used are shown in Table 1. For RT-PCR analysis, cDNA synthesis was performed for 30 min at 45 °C, and the products were denatured for 2 min at 94 °C. PCR amplification was carried out for 38 cycles (denaturation for 60 s at 94 °C, followed by primer annealing for 90 s at 55 °C, and extension for 120 s at 72 °C). GAPDH was used as a loading control.

2.5. Quantitative real time RT-PCR

To validate gene expression changes, quantitative real time RT-PCR analysis was performed with an Applied Biosystems Prism 7900HT Sequence Detection System according to manufacturer's instructions (Applied Biosystems Inc., California, USA) for runx2 and osterix. TaqMan Gene Expression Assays for runx2 (assay identification number Mm00501580_m1) and osterix (assay identification number Mm00504574_m1) were inventoried products (Applied Biosystems Inc.). Mouse GAPDH gene was used as endogenous control (assay identification number Mm03302249_g1). After reaching confluence, cells were incubated in conditioned medium for 14 days. Total RNA (2 μg) was extracted from cells using ISOGEN (Nippon Gene). Reverse-transcriptase reaction was performed with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems Inc.). The thermal cycler conditions were as follows: step 1 for 60 min at 37 °C and step 2 for 5 min at 95 °C. PCR amplification with real-time detection was performed with TaqMan Gene Expression Master Mix (Applied Biosystems Inc.) and total RNA of 100 ng/μl. Thermal cycling conditions comprised an initial UNG incubation at 50 °C for 2 min, AmpliTaq Gold DNA polymerase activation at 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. All samples were performed in triplicate. Amplification data were analyzed with an Applied Biosystems Prism Sequence Detection Software version 2.1 (Applied Biosystems Inc.). To normalize the relative expression of the genes of interest to the GAPDH control, standard curves were prepared for each gene mentioned above and the GAPDH in each experiment.

2.6. Western blot analysis

To detect cyclin D1, cdk4, and cdk6, cells were plated at a density of 5×10^5 cells in a 100-mm-diameter dish and allowed to proliferate for 24 h. Their growth was then arrested by incubation for 48 h in α-MEM containing 0.5% FBS. Growth-arrested cells were

Table 1
Mouse primers for RT-PCR.

	Forward	Reverse
α1	TCTCAAGCAAAAAGTGGTCG	ATTCCGAGATCTGCCTGTCT
α2	CTCCCATCCTGCTTTCCAG	GTTTGAACAGGCGGTCCTC
α3	GTGAATTCTTCAGCCGTGCAGACTCCA	ATAAGCTTGGCAACGTACTTCCAATCATC
α4	GTGAATTCCACAGGTCGTACACGGGTCG	ATAAGCTTGCGAGCCCGGCATCTTGAGT
α5	AGTGGGGCTGGACCTAAATCTCG	CAAAAAGCCCTAAAGTCCCAATGA
α6	CTTTGTCACGCTGTCCAT	GCCTCCTTTGTCTTGTCC
α7	ACAGTACTTCGCCAGCACCA	AAACCATGCACACCAATTCA
α9	ACAAGGCCACCAACTCCA	ACCAACCCACTCCTCCTCTT
α10	TCTGACCTCACAACCCACAA	TCCTGTCTCAGCCTCCATGT
β1	TGATGTGGTGCTGCTGAACAA	CAACGTCGAAATTTCCGTCAT
β2	GTGAATTCAGGGCGAGGCGGTTTTCTT	ATAAGCTTGCGTACGCCATCCACTGCT
β3	GTGAATTCTGGGTGAAGAGGCTGTT	ATAAGCTTATCGCTGGCGGGAGTCTGTT
β4	GTGAATTCCATGGCATCCTGGGTCAAG	ATAAGCTTCTGGGGAGGCCTGCTGTGT
γ	GATGCAATGGTGCGACTATCGC	GCCTCCGGGTCAATGAAGATCC
δ	CTGCCAGTCGAA	CTGCTGGGAAATCCTAGGCACACTTGAG
ε	ATTGAAGAGCTTAGCCTGTA	TACACCTGCAAAATCGTCCT
M1	GCAGCAGCTCAGAGAGGTCACAG	GATGAAGGCCAGCAGGATGG
M2	GCGGATCCTGTGGCCAACCAAGAC	CGAATTCACGATTTTGCGGGCTA
M3	AAGGCACCAAACGCTCATCT	GCAAACCTCTTAGCCAGCGT
M4	AGCCGCAGCCGTGTTCACAA	TGGGTTGAGGGTTCGTGGCT
M5	GTCTCCGTCATGACCATACTCTA	CCCGTTGTTGAGGTGCTTCTAC
ChAT	CCTGCCAGTCAACTCTAGCC	TCAGGGCAGCCTCTCTGTAT
AChE	TTGGAGTCTCGAGGGTCATT	GGACGAGGGCTCCTACTTTC
VAChT	ATGATCGCCGACAAGTATCC	CCATTGTGTGCTTCATCC
CHT1	CATCCTCAGCCACCTATGCT	TGGATACCCGTAGGCAGTCT
Collagen 1 a1	TTGATCTGTATCTGCCACAA	GCTGATTTTTCATCATAGCC
Osteocalcin	TGCTTGTGACGAGCTATCAG	GAGGACAGGGAGGATCAAGT
GAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC

treated with growth medium for the indicated times in the presence or absence of ACh. The analysis was performed as described previously [21]. Briefly, cells were rinsed with ice-cold PBS and lysed with a lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 8.0], 10 μg/ml aprotinin, 0.1 M NaF, 2 mM Na_3VO_4, and 1 mM PMSF). The lysates were incubated on ice for 15 min and centrifuged at 15,000×*g* for 10 min at 4 °C. The protein content was quantified by the Lowry method (BIO RAD). Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (BIO RAD). After the blocking of nonspecific binding by soaking the filters in 5% skim milk (Sigma–Aldrich), the desired proteins were immunodetected with their respective antibodies, followed by visualization with the use of an ECL Plus Western blotting detection system (GE Healthcare), according to manufacturer's instructions. The bands were scanned with a LAS-3000mini luminescent image analyzer (Fuji Film, Tokyo, Japan). β-Actin was used as a loading control.

2.7. Measurement of ALP activity

Cells were plated at a density of 2×10^4 cells in 24-well plates. After reaching confluence, cells were incubated in conditioned medium with stimulants (ACh, CCh, Mus, and Nic) at the indicated doses for seven days. To assess the effects of inhibitors (Mec and Atr), cells were pre-treated with inhibitors at the indicated doses, which were added 1 h before treatment with stimulants. The ALP activity was assayed (Wako) as described previously [22] and normalized by the amount of protein, determined using the Lowry method (BIO RAD). The measurements are expressed as the means of at least three independent experiments, with each data point based on four replicates.

2.8. Statistical analysis

The data are presented as means ± S.D. Statistical significance was assessed by Student's *t*-test using SPSS 14.0J. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. ACh, CCh, Nic, and Mus induce cell proliferation via AChRs in osteoblasts

Since we previously showed that Nic, an agonist of nAChRs, induced proliferation of osteoblasts [23], we examined whether CCh (an analog of ACh) at the indicated doses promoted S phase entry in MC3T3-E1, assessed by BrdU incorporation assay. CCh significantly increased the osteoblast proliferation rate (Fig. 1A). We analyzed the effects of ACh, CCh, and antagonists such as Mec and Atr. ACh and CCh significantly induced cell proliferation, and the proliferative effects of ACh and CCh were abrogated by the nAChRs and mAChRs antagonists Mec and Atr (Fig. 1B and C). We then tested whether Nic and Mus promoted S phase entry via each receptor in MC3T3-E1. Nic and Mus significantly induced cell proliferation, and the proliferative effects of Nic and Mus were abrogated by Mec and Atr (Fig. 1D).

Next, we examined whether these drugs could promote cell proliferation in murine pOB. ACh, Nic, and Mus significantly induced cell proliferation, and the proliferative effects of ACh, Nic, and Mus were abrogated by Mec and Atr in pOB (Fig. 1E and F). These results suggest that agonists of AChRs promote S phase entry in murine osteoblasts.

3.2. ACh up-regulates cyclin D1 expression in MC3T3-E1 cells

Next, we studied the role of signal transduction in the proliferative effects of ACh. Since Nic up-regulates cyclin D1 expression in MC3T3-E1 [23], the contributions of cyclin D1, cdk4, and cdk6 were examined by Western blot analysis. The expression of cdk4 and cdk6 did not change during serum stimulation in the presence or absence of ACh. The amount of cyclin D1 showed little change until 48 h in the absence of ACh, but increased dramatically until 48 h in the presence of ACh (Fig. 2). These results suggest that ACh promotes cell cycle progression by up-regulating cyclin D1.

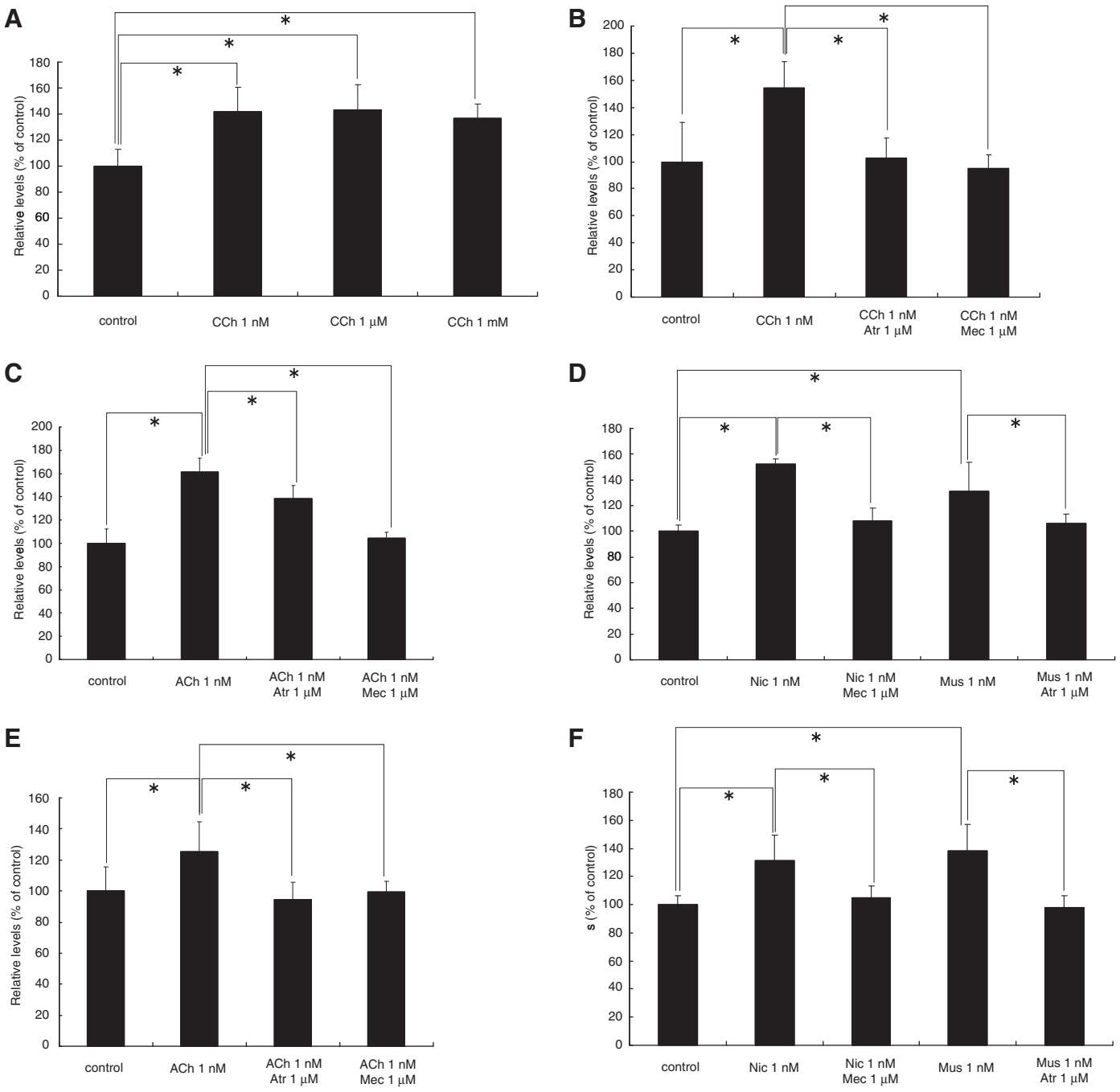

Fig. 1. Agonists of AChRs induce cell proliferation in osteoblasts. We used MC3T3-E1 in (A–D) and pOB in (E) and (F). (A) Cells were treated with CCh at various concentrations (1 nM, 1 μM, 1 mM). (B) Cells were treated with 1 nM CCh in the presence or absence of Mec (1 μM) or Atr (1 μM). (C) Cells were treated with 1 nM Nic or 1 nM Mus in the presence or absence of Mec (1 μM) or Atr (1 μM). (D) Cells were treated with 1 nM ACh in the presence or absence of Mec (1 μM) or Atr (1 μM). (E) Cells were treated with 1 nM Nic or 1 nM Mus in the presence or absence of Mec (1 μM) or Atr (1 μM). (F) Cells were treated with 1 nM ACh in the presence or absence of Mec (1 μM) or Atr (1 μM). Error bars represent ± S.D. * $P < 0.05$ as compared with control.

3.3. ACh, CCh, and Mus reduced ALP activity in osteoblasts

We previously reported that Nic reduced ALP activity [23]. We therefore investigated whether ACh, CCh, and Mus directly affect ALP activity in MC3T3-E1 cultured in conditioned medium. As shown in Fig. 3A, ALP activity in MC3T3-E1 was significantly reduced by Nic and Mus in dose-dependent manners on day 7. ACh also reduced ALP activity, and the inhibitory effect of ACh was abrogated by the nAChRs and mAChRs antagonists Mec and Atr in MC3T3-E1 on day 7 (Fig. 3B). In pOB, CCh reduced ALP activity on day 7 (Fig. 3C). We also tested whether ACh could reduced mineralization, but ACh had no effect (data not shown). Thus, our data indicate that agonists of AChRs reduced ALP activity in osteoblasts.

3.4. AChE, VAChT, ChAT, and CHT1 mRNAs were detected in osteoblasts

Next, we examined the mRNA expression of cholinergic components such as AChE, VAChT, ChAT, and CHT1 during osteoblastic differentiation. To confirm osteoblastic differentiation, we analyzed the expression of osteogenic markers, such as osteocalcin, runx2 and osterix. We detected up-regulation of osteocalcin mRNA during osteoblastic differentiation, while col1a1 and GAPDH mRNA

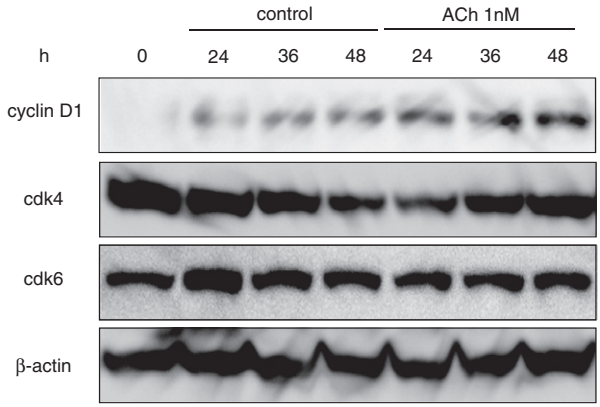

Fig. 2. ACh induces cyclin D1 expression in MC3T3-E1. Growth-arrested MC3T3-E1 cells were stimulated with serum in the presence or absence of 1 nM ACh. Cell lysates were collected at 24, 36, and 48 h.

showed little change by RT-PCR (Fig. 4A). We also detected significant up-regulation of runx2 and osterix mRNA during osteoblastic differentiation using real time RT-PCR (Fig. 4B).

As shown in Fig. 4C, AChE, VAChT, ChAT, and CHT1 mRNAs were detected in both immature and mature osteoblasts. CHT1 mRNA was expressed in murine pOB, but not in MC3T3-E1. Moreover, pOB showed slightly increased mRNA expression of AChE, VAChT, ChAT, and CHT1. These results suggest that the production of cholinergic components is increased during osteoblastic differentiation.

3.5. Cholinergic receptors alter their subunits/subtypes during osteoblastic differentiation

It was previously demonstrated that several factors which promote neuronal differentiation induce differential regulation of nAChR subunit expression in neuronal cells [24]. So we examined the expression of mRNAs for mAChRs and nAChRs in MC3T3-E1 and pOB before and after differentiation induction by RT-PCR. As shown in Fig. 4G, β4-nAChR mRNA was expressed in pre-osteoblasts. After differentiation induction, M1, M2 and M4-mAChRs and α1, α6, α7, β1, δ and ε-nAChRs mRNAs were up-regulated (Fig. 4D–H).

Next, we examined the expression of mRNAs for mAChRs and nAChRs in MC3T3-E1 and pOB treated with ACh in conditioned medium. ACh treatment in conditioned medium for 14 days reduced the expression of M2 and M4-mAChRs and α6 and α7-nAChRs mRNA compared to the level in the absence of ACh (Fig. 4D–H). These data indicate that subunits/subtypes of AChRs alter during osteoblastic differentiation (Fig. 5).

4. Discussion

In this study, we obtained several interesting findings concerning the relation between osteoblasts and ACh. First, ACh promotes cell cycle progression in osteoblasts. Second, ACh decreases ALP activity during osteoblastic differentiation. Third, mature osteoblasts express specific subunits/subtypes of ACh receptor. Finally, mature osteoblasts express molecules that produce and degrade ACh during osteoblastic differentiation.

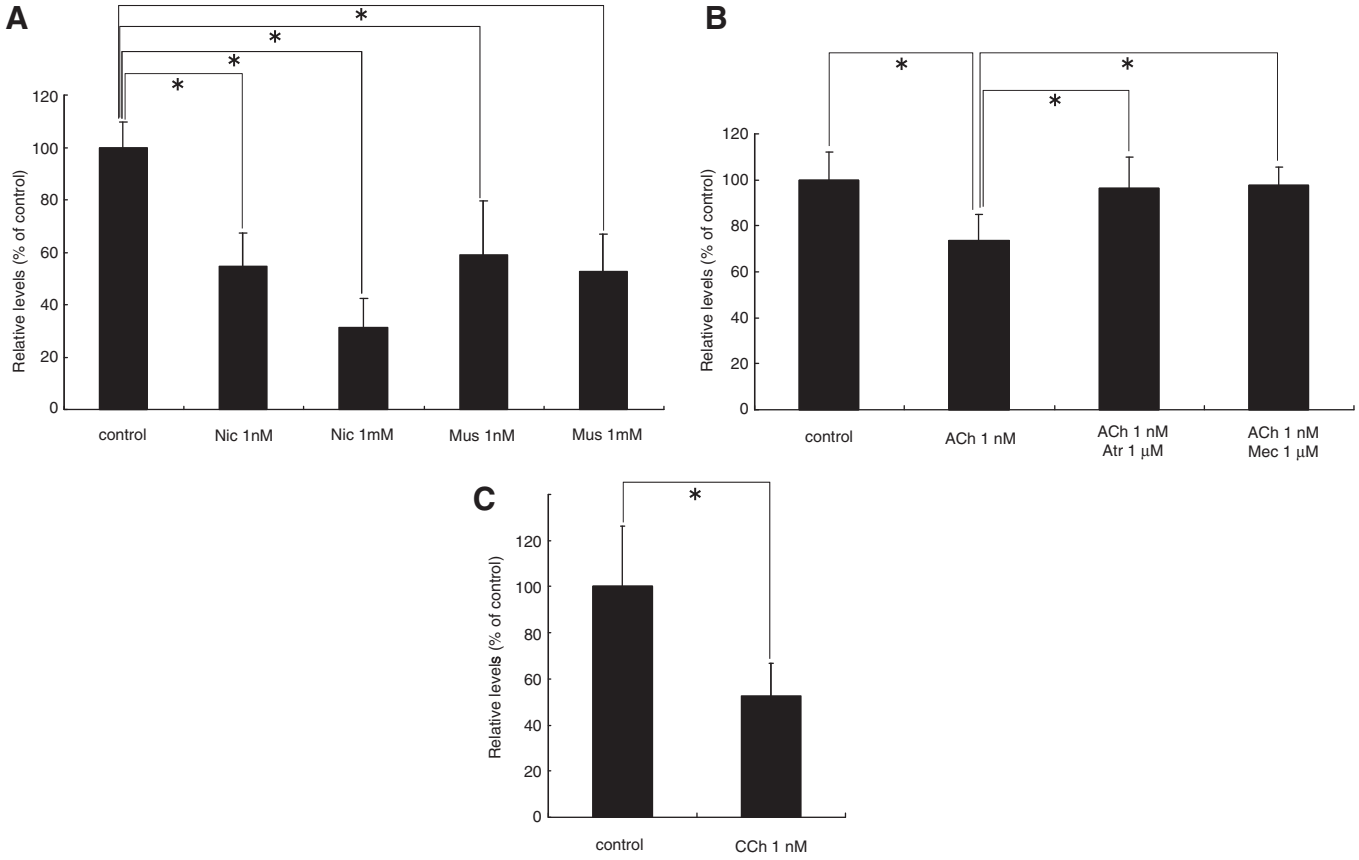

Fig. 3. Agonists of AChRs reduce ALP activity in osteoblasts. We used MC3T3-E1 in (A) and (B) and pOB in (C). (A) Cells were treated with Nic at various doses (1 nM and 1 μM) and Mus at various doses (1 nM and 1 μM). (B) Cells were treated with 1 nM ACh in the presence or absence of Mec (1 μM) or Atr (1 μM). (C) Cells were treated with 1 nM CCh. Error bars represent ± S.D. * $P < 0.05$ as compared with control.

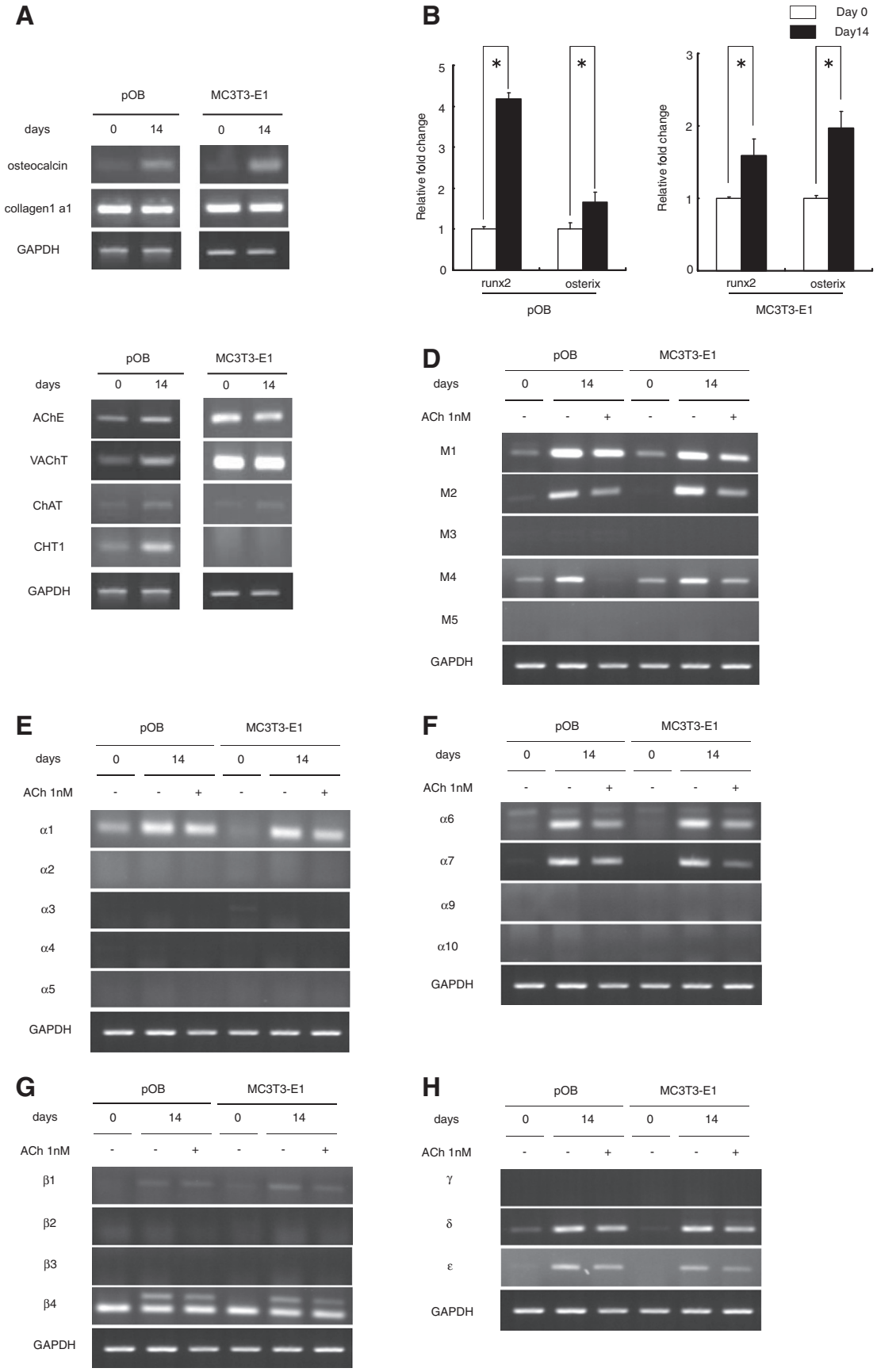

Fig. 4. Osteoblasts express mRNA for cholinergic receptors and cholinergic components. mRNA expression after incubation with conditioned medium in pOB and MC3T3-E1 for 14 days. (A) Osteocalcin and col1a1 detected by RT-PCR, (B) relative fold change of runx2 and osterix mRNA detected by real time RT-PCR, (C) cholinergic components detected by RT-PCR. (D–H) mAChRs and nAChRs detected by RT-PCR in the presence or absence of ACh (1 nM).

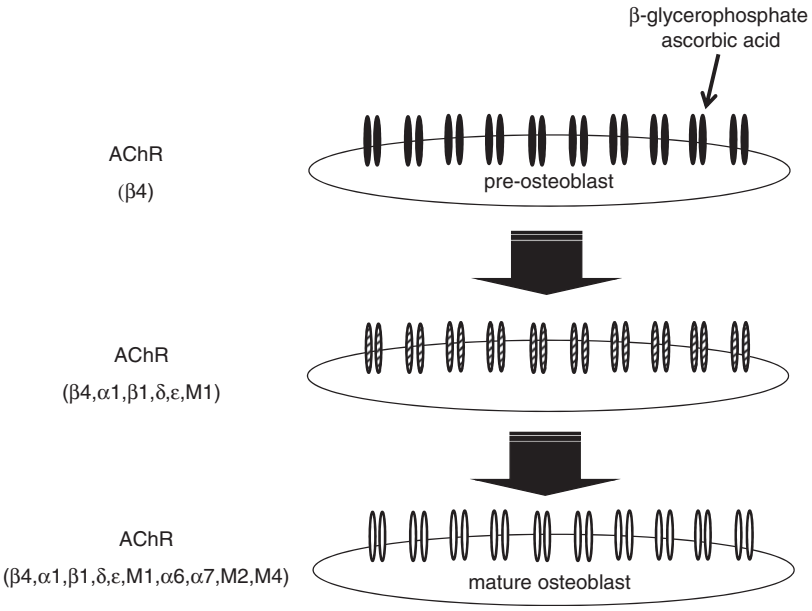

Fig. 5. The expression of AChRs during osteoblastic differentiation. Cells stimulated with β-glycerophosphate and ascorbic acid change subunits/subtypes of AChRs and responsiveness to ACh.

Our results clarified the expression of AChRs in pre-osteoblasts and differentiated osteoblasts. β4-nAChR was expressed intensely in pre-osteoblasts. This subunit is widely expressed in the central and peripheral nervous systems. Within the central nervous system, transcripts for β4 subunit expression are restricted [25]. We detected α1, α6, α7, β1, δ and ε-nAChRs in differentiated osteoblasts. Interestingly, the α1, β1, δ and ε subunits are expressed in the muscular system but not in the nervous system, whereas the α6 and α7 subunits are expressed in the nervous system but not in the muscular system [26]. The nAChRs of mature osteoblasts have properties of the nervous and muscle systems. M1 and M4-mAChRs expression was weakly detected in pre-osteoblasts, whereas M1, M2 and M4-mAChRs expression was strongly detected in differentiated osteoblasts. Walker et al. previously demonstrated the presence of the α4-nAChR subunit in human pOB, and Katono et al. showed that Nic increased the expression of the α7-nAChR subunit in a human osteosarcoma cell line [27,28]. We found no expression of the α4-nAChR subunit in murine osteoblasts, suggesting a species difference between mice and humans. A number of researchers have reported the role of α7-nAChR and the M1-mAChR in cell proliferation [29–31]. Available evidence suggests that α7-nAChR and M1-mAChR may be involved in the cellular functions of osteoblasts. In contrast to Nic, ACh did not reduce mineralization, suggesting that the mAChRs pathway plays a role in the regulation of mineralization [23].

It seems to be a discrepancy that ACh treatment reduces ALP expression in osteoblasts while differentiation induction promotes the secretion of ACh. Here we propose a following model to explain the phenomena. The action of ACh on osteoblasts changes with differentiation, since AChRs alter their subunits/subtypes composition during osteoblastic differentiation. Once ascorbic acid and β-glycerophosphate promotes differentiation of pre-osteoblastic cells which express only β4-nAChR, osteoblasts begin to express M1, M2, M4, α1, α6, α7, β1, δ and ε-AChRs and produce ACh in mature phase. In our model, we conjecture that ACh inhibits osteoblastic differentiation via β4-nAChR and particular subunits/subtypes of receptors, i.e., M2, M4, α6 and α7-AChRs, block inhibitory signaling of differentiation by ACh.

Many non-neuronal cells are equipped with recycling pathway of ACh [12]. AChE has been found to be essential for osteogenesis as well as chondrogenesis [32,33]. AChE may play a part not only in the degradation of ACh, but also in the differentiation of bone and cartilage during development. We detected a recycling system of ACh in osteoblasts. While ChAT, CHT1, AChE, and VAChT mRNAs increased during differentiation of murine pOB, only ChAT mRNA increased during differentiation of MC3T3-E1 and AChE and VAChT mRNA were strongly expressed before differentiation induction in MC3T3-E1. The reason for this difference is unclear, but we conjecture that cell immortalization may be related to the high expression of these molecules. The synthesis of plasma membrane lipids requires the uptake of choline by choline transporters. CHT1 is a choline transporter expressed by only a few types of non-neuronal cells [34]. MC3T3-E1 does not express CHT1 mRNA, but pOB do. Perhaps another choline transporter is expressed in MC3T3-E1.

We speculate that ACh plays the following role in osteoblastic differentiation: the properties of cholinergic receptors change in pre-osteoblastic cells, increasing the production of ACh during differentiation; then ACh acts on pre-osteoblastic cells to promote cell cycle progression by up-regulating cyclin D1 and decreasing ALP activity. One nanomole of ACh is sufficient for a pharmacological response. Our present results are consistent with the data that ACh in the nanomolar concentration range act on macrophages [35].

Cholinergic components and AChRs antagonist affect cartilage and bone metabolism in vivo. In membrane bones of embryonic chicks paralyzed by the in ovo injection of D-tubocurarine, an inhibitor of nAChRs, secondary cartilage failed to differentiate [36]. Moreover, impaired AChE gene expression in thanatophoric dysplasia, considered one of the most severe forms of chondrodysplasia, has been described [32]. These results suggest that cholinergic signaling may play a vital role in bone and cartilage metabolism in vivo. However, bone metabolism has yet to be analyzed in AChR knockout mice. Whether parasympathetic innervation contributes to periosteal development is unclear; the parasympathetic nervous system most likely regulates bone remodeling, but this remains speculative. Further experiments are necessary to unravel the relation between the parasympathetic nervous system and bone remodeling.

Acknowledgements

The authors would like to thank Mariko Hayakawa for excellent technical assistance. This work was supported in part by a Grant-in-Aid (18791517) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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