Age-related decreases in stimulatory G protein-coupled adenylyl cyclase activity in osteoblastic cells

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Age-related decreases in stimulatory G protein-coupled adenylyl cyclase activity in osteoblastic cells. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E776-E781; 1997.—In this study we examined parathyroid hormone (PTH)-, forskolin (FSK)-, and cholera toxin (CTX)-stimulated adenosine 3',5'-cyclic monophosphate (cAMP) accumulation in rat osteoblastic cells (ROB) isolated from young (4 mo), mature (12 mo), and old (24–28 mo) male rats. Exposure to PTH increased cAMP accumulation in a concentration-dependent manner in all ROB cells examined. However, the maximum response in ROB from young rats was threefold greater than the maximum response in those from mature and old rats. Exposure to FSK also stimulated cAMP accumulation in a concentration-dependent manner, but there were no significant differences in responsiveness among ROB isolated from young, mature, and old rats. Exposure to CTX resulted in a dramatic concentration-dependent increase in cAMP in ROB from young rats but only a modest increase in ROB from mature and old rats. PTH binding kinetics were similar in ROB from rats in each age group. These data suggest an age-related defect in stimulatory G protein coupling to adenylyl cyclase, which contributes to decreased osteoblastic responsiveness to PTH.

parathyroid hormone; signal transduction; rat; bone cell; forskolin; cholera toxin

BONE FORMATION decreases with age, suggesting that decreased osteoblast activity may contribute to age-related osteopenia (19). Osteoblast activity is determined to a large extent by the ability of the cells to adapt to changes in the extracellular milieu, especially changes in hormonal signals. Considering that many hormonal signals are transduced at the cell membrane through the generation of second messengers [adenosine 3',5'-cyclic monophosphate (cAMP) and cyclicositol calcium], a disruption in this process could catalyze alterations in the ability of osteoblasts to adapt to hormonal signals and thereby decrease their activity.

There is considerable evidence that hormonal regulation of signal transduction diminishes with age (17). Studies have demonstrated an age-related decline in parathyroid hormone (PTH)-stimulated adenylyl cyclase in both rat kidney slices (2) and renal cell membranes (11, 12). Also observed is a decrease in β-adrenergic-stimulated adenylyl cyclase in rat erythrocytes (6) and brown adipose tissues (27) and in human lymphocytes (14) and in dopamine-stimulated adenylyl cyclase in rat striatum (22). Cytosolic calcium concentration ([Ca^2+]i) in cerebral cortical synaptosomes from aged rats has been shown to be increased (16) or unchanged (10) compared with that in synaptosomes from younger rats. Additionally, compared with those of young mice (4 mo), T lymphocytes from old mice (24 mo) have higher basal [Ca^2+]i, but less of an increase in [Ca^2+]i, in response to concanavalin A (21). As regards the musculoskeletal system, in vitro studies on chondrocytes have shown an age-dependent decrease in basal [Ca^2+]i (4). Unfortunately, there have been only a few studies that have examined age-related changes in signal transduction pathways in bone cells, and their results have been contradictory. For instance, several investigators have reported that PTH-stimulated cAMP accumulation is reduced in rat, mouse, and human osteoblastic cells in vitro (9, 15, 29), whereas Pfilschifter et al. (20) have reported an age-related increase in PTH-stimulated cAMP accumulation in human osteoblastic cells. Furthermore, none of these studies have systematically investigated the mechanism underlying changes in PTH responsiveness with age.

To address this issue we examined PTH regulation of adenylyl cyclase activity in primary cultures of rat osteoblasts (ROB) isolated from young (4 mo old), mature (12 mo old), and old (24–28 mo old) male Fisher 344 rats. To examine the mechanisms underlying any age-related changes we found in signal transduction, we also examined forskolin- and cholera toxin (CTX)-stimulated adenylyl cyclase activity and PTH binding kinetics in ROB.

METHODS

Reagents. Rat PTH fragment 1—34 [rPTH-(1—34)] and [Tyr^36]PTH related protein ([Tyr^36]PTHrP)-(1—36) were purchased from Bachem (Torrance, CA). cAMP radioimmunoassay (RIA) kits were purchased from Incstar (Stillwater, MN). Dulbecco’s modified Eagle’s medium (DMEM), penicillin, and streptomycin were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hydione Laboratories (Logan, UT). Forskolin and CTX were purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were of tissue culture grade.

Primary culture of ROB. ROB were isolated from the tibiae and femora of 4-, 12-, and 24- to 28-mo-old male Fisher 344 rats as previously described (8). Briefly, subperiosteal osteoblastic cells were isolated by sequential collagenase digestion at 37°C. Second digestion cells were collected by centrifugation and placed in T-25 tissue culture flasks and fed with DMEM, 20% FBS, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. The cells were allowed to reach confluence and then were subcultured into culture dishes appropriate for each experiment. We have previously demonstrated that cells
isolated in this manner express phenotypic characteristics of osteoblastic cells (8).

Quantification of cAMP accumulation in ROB. ROB cells were grown to confluency or, in some cases, 5 days postconfluence in six-well multiwell plates. PTH stimulation was carried out in culture media to which had been added 1 mM isobutylmethylxanthine (IBMX). The cells were exposed for 15 min at 37°C to 10^{-6} to 10^{-10} M rPTH-(1—34), 10^{-3} to 10^{-7} M forskolin, or 10^{-6} to 10^{-10} M CTX. Cells grown 5 days postconfluence were exposed to only 10^{-6} to 10^{-10} rPTH-(1—34). The reaction was stopped by the addition of boiling sodium acetate buffer (0.05 M + 1 mM IBMX, pH 5.1). Cells were scraped from the wells and transferred with the buffer to glass tubes. Each well was washed with another 0.5 ml of buffer, which was pooled with the scraped cells. The glass tubes were placed in a boiling water bath for 5 min and subsequently transferred to ice for 10 min. The cold tubes were centrifuged at 1,000 g for 10 min, and the supernatant was frozen at −20°C for later analysis of total cAMP (cells and media) by RIA.

PTH/PThrP receptor binding. Binding studies were performed as previously described (28). Briefly, cells were plated in 24-well dishes and grown to confluence. Previous studies with primary osteoblastic cells indicating a temporal pattern of PTH/PThrP receptor expression, binding, and PTH-stimulated cAMP activity formed the basis for selection of time points for receptor binding studies (18). The cells were cultured for 1, 3, or 5 days in media supplemented with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate before receptor binding assays. Each assay, performed in triplicate, included the addition of 25,000 counts/min of moniodinated 125I-labeled PThrP-(1—36) in addition to varying concentrations of nonradioactive PThrP-(1—36), the ligand binding equivalent of PTH in ROS 17/2.8 cells, the removal of unbound peptide, and cell lysis. Cell suspensions were counted by scintillation, and specific activity was calculated by subtracting the radioactivity bound in the presence of excess (1 M) unlabeled ligand from those with tracer only. In each experiment the ratios of bound to bound in absence of competitor were computed as means ± SE and number of receptors per microgram of DNA calculated based on Scatchard maximum binding capacity values.

RNA isolation and Northern blot analysis. Total RNA was extracted by previously published methods (7) from cells on 100-mm dishes in a manner identical to that used for cAMP assays. Total RNA (20 µg) was denatured, separated, and transferred to a nylon solid support. Blots were hybridized with [α-32P]dCTP-labeled probes of either a 1.8-kilobase pair (kb) BamHI I-NcoI fragment of the rat PTH/PThrP receptor cDNA, a 1.2-kbp EcoR I fragment of rat osteopontin cDNA, a 1.8-kbp PstI-Hind III fragment of the chicken type I collagen cDNA or a 1.4-kbp PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Hybridizations and washes were performed according to manufacturer’s instructions (Schleicher & Schuell), followed by exposures for various time periods to Kodak X-Omat AR film with a Cronex intensifying screen. The abundance of specific mRNAs was quantified using a Phosphorimage densitometer. Data were normalized to GAPDH mRNA abundance.

Alkaline phosphatase assay. A histological stain was used to evaluate the presence of alkaline phosphatase activity on the surface of cells. Cells on 12-well culture plates were rinsed with distilled water, fixed briefly, stained according to manufacturer’s instructions (Sigma), and visualized under light microscopy.

Statistics. The concentration-dependent effect of various reagents was analyzed by one-way analysis of variance followed by the Student-Neuman-Keuls test. Significance was accepted at P < 0.05.

RESULTS

Basal cAMP levels were significantly (P < 0.05) lower in ROB isolated from young rats (3.4 ± 0.9 nmol/g protein, means ± SE) relative to ROB isolated from mature (9.7 ± 2.0 nmol/g protein) or old (8.9 ± 1.5 nmol/g protein) rats. In cells grown to confluence, exposure to PTH for 15 min increased total cAMP accumulation in a concentration-dependent manner in ROB from young rats (Fig. 1). However, the cAMP response to PTH in ROB from mature and old rats was dramatically reduced relative to ROB from young rats. PTH-stimulated cAMP accumulation in ROB isolated from young rats was significantly greater than control at concentrations above 10^{-10} M. In ROB isolated from young rats, maximal stimulation of cAMP occurred at 10^{-8} M. In ROB from mature rats, PTH-stimulated cAMP accumulation was significantly greater than control at concentrations of 10^{-9} M and above. However, the cAMP levels plateaued at 10^{-9} M and did not significantly increase at higher concentrations. The cAMP response to PTH was similar in ROB cells isolated from old rats. At PTH concentrations of 10^{-10} M and greater, cAMP accumulation was significantly greater in ROB from young rats relative to mature rats. PTH-stimulated cAMP was similar at all concentrations tested in ROB isolated from mature and old rats. Similar results were obtained from cells cultured five days postconfluence (data not shown).

To determine whether a defect in adenylate cyclase per se was responsible for the age-related attenuation of PTH-stimulated cAMP in ROB cells, we examined forskolin-stimulated cAMP accumulation. A 15-min exposure to forskolin resulted in a concentration...
dependent increase in cAMP accumulation in ROB isolated from young, mature, and old rats (Fig. 2). There were no statistically significant differences between forskolin-stimulated cAMP accumulation in ROB isolated from young, mature, or old rats.

To examine whether an age-induced defect in the catalytic subunit of Gs protein contributed to the attenuation of PTH-stimulated cAMP with age, we quantified CTX-stimulated cAMP accumulation. Cells isolated from young, mature, and old rats exposed to CTX displayed a concentration-dependent increase in cAMP (Fig. 3). In ROB isolated from young rats, CTX significantly increased cAMP, relative to control, at all concentrations examined and was maximal at $10^{-6}$ M. On the other hand, in ROB isolated from mature and old rats, exposure to CTX resulted in a moderate, relative to young ROB, concentration-dependent increase in cAMP. In ROB from mature and old rats, exposure to CTX resulted in maximal stimulation of $\sim$2-fold, whereas in young ROB maximal stimulation of $\sim$10-fold was attained.

To determine whether decreased hormonal responsiveness in ROB isolated from old rats could be due to reductions in PTH binding or receptor availability, radiolabeled binding studies were performed on ROB cells that had been cultured for 1, 3, or 5 days postconfluence. The comparison of young, mature, and old cells at each time point was similar. Data from the 5-day culture groups are presented in Table 1. Radioiodinated PTHrP-(1–36), a ligand with binding kinetics for the PTH/PTHrP receptor equivalent to PTH (1), was found to specifically bind to the PTH/PTHrP receptor with $50\%$ effective concentration ($EC_{50}$) and numbers of receptors per microgram of DNA similar to previously reported results (18). From nonlinear regression of competition binding curves for PTHrP, the $EC_{50}$ and number of specific binding sites were obtained. Cell isolates from young, mature, and old rats examined bound PTHrP with similar affinities. Scatchard transformation revealed that ROB isolated from young, mature, and old rats expressed similar numbers of PTH/PTHrP receptor per cell. Furthermore, ROB cells from young, mature, and old rats had detectable mRNA for the PTH/PTHrP receptor by Northern blot analysis (Fig. 4). These data confirm that ROB isolated from young, mature, and old rats contain similar levels of PTH/PTHrP receptors that bind PTH analogs.

Osteoblastic characteristics include expression of type I collagen, osteopontin, and PTH/PTHrP receptor mRNA; therefore, Northern blot analysis was performed to identify any loss of these osteoblastic phenotypic markers associated with the age of the rat from which ROB were isolated. ROB isolated from young, mature, and old rats demonstrated expression of type I collagen, osteopontin, and PTH/PTHrP receptor mRNA as assessed by quantification of total cellular RNA normalized to GAPDH (Fig. 4). Another characteristic

### Table 1. PTH/PTHrP receptor number and ligand binding affinity in osteoblastic cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>$EC_{50}$, nM</th>
<th>No. of Receptors/µg DNA ($\times 10^{9}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young ROB</td>
<td>0.6 ± 0.36</td>
<td>2.3 ± 1.6</td>
</tr>
<tr>
<td>Mature ROB</td>
<td>0.47 ± 0.10</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>Old ROB</td>
<td>0.21 ± 0.11</td>
<td>5.1 ± 1.9</td>
</tr>
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Data are presented as means ± SE of 3 experiments/cell line. Binding assays were performed on cells plated in 24-well dishes and grown to confluence. Monoiodinated $^{125}$I-labeled parathyroid hormone (PTH)-related protein (PTHrP)-(1–36) was added in addition to varying concentrations of nonradioactive PTHrP-(1–36). Original data were subject to nonlinear regression of competition binding curves to obtain $50\%$ effective concentration ($EC_{50}$) values. Numbers of receptors per cell were calculated from maximum binding capacity after Scatchard transformation. ROB, rat osteoblastic cells.
of the osteoblast phenotype is the presence of alkaline phosphatase activity, a marker for osteoblastic differentiation that is associated with mineralization. We found that alkaline phosphatase abundance was similar in ROB isolated from young, mature, and old rats (data not shown).

DISCUSSION

Previous studies on osteoblastic cells isolated from rats and humans have demonstrated that PTH-stimulated cAMP accumulation decreases as a function of age of the animal from which the osteoblasts were isolated (9, 15, 29). However, the mechanism underlying this decreased hormonal responsiveness has not been extensively studied. Our results confirm these previous studies and suggest that a decreased sensitivity to PTH occurs in osteoblastic cells isolated from aged rats. There are several possible explanations for this phenomenon.

One possibility is that the catalytic component of adenylate cyclase is dysfunctional in osteoblastic cells isolated from older rats. However, we found that forskolin, which directly activates the catalytic component of adenylate cyclase, stimulated cAMP accumulation to a similar degree in ROB isolated from young, mature, and old rats. These findings are consistent with previous studies from human osteoblasts (29) and rat renal cells (12) and suggest that the catalytic component of the adenylate cyclase complex is unaffected by aging (12).

It is also possible that the age-related decrease in PTH-stimulated adenylate cyclase activity we observed is a result of a decrease in the activity of Gs protein-coupled adenylate cyclase activity as a function of age. To examine this possibility, we exposed cells to CTX, which catalyzes the ADP-ribosylation of Gs, thus facilitating its activation of adenylate cyclase. Indeed, we found that CTX-stimulated cAMP accumulation was dramatically attenuated in ROB isolated from mature and old rats, relative to young rats. Wong et al. (29) also reported that CTX-stimulated cAMP accumulated in human osteoblastic cells isolated from individuals >40 yr old, relative to those isolated from individuals <40 yr old. However, the difference did not reach statistical significance. Thus our data suggest an age-related defect in Gs protein-coupled adenylate cyclase activity in osteoblastic cells. There was a trend toward increased PTH receptor binding affinity as a function of age. Although this change did not reach statistical significance, it is tempting to speculate that this reflects an attempt of ROB from older rats to compensate for the uncoupling of receptor effector systems.

Another possibility that may explain our data is that the number of fully differentiated osteoblastic cells may decrease as a function of age. This is suggested by the finding that the number of stromal preosteoblastic cells and their ability to commit properly into the osteoblast lineage are decreased in 24-mo-old rats relative to 6-mo-old rats (23) and the observation of impaired osteoblastogenesis in the osteopenic senescence accelerated mouse SAMP6 (13). Furthermore, the expression and biological activity of the PTH/PTHrP receptor is associated predominantly with an active matrix-producing osteoblastic cell and not with less or more differentiated cells (18). Thus it is possible that the decreased cAMP responsiveness we observed in ROB from older rats was due to a decrease in the number of mature, fully differentiated osteoblasts in the isolates from older rats. However, if this were the case, one would expect that, in older rats, the PTH dose-response curve would display a concentration-dependent effect of PTH, but with lower cAMP accumulation at each PTH concentration, rather than the relatively flat dose-response curve we observed. Furthermore, a decrease in mature osteoblasts alone could not explain the age-related decrease in CTX-stimulated cAMP accumulation, since CTX would be expected to increase adenylate cyclase activity in preosteoblastic cells as well as in more mature osteoblasts.

It is also possible that osteoblastic cells isolated from rats of different ages express different phenotypes and thus different cAMP responses to PTH. However, we believe that this is unlikely, since PTH binding kinet-
ics, the phenotypic characteristic most relevant to our studies, are similar in osteoblastic cells isolated from young, mature, and old rats. Furthermore, osteoblastic cells from young, mature, and old rats express type I collagen, osteopontin, and PTH/PTHrP receptor mRNA as well as abundant alkaline phosphatase activity. Taken together, these data suggest that the osteoblastic phenotype was maintained in cells isolated from young, mature, and old rats.

The age-related attenuation of PTH-stimulated cAMP accumulation we observed is strikingly similar to age-related changes in PTH effects on renal cells reported by Hanai et al. (11, 12). Those investigators showed that, compared with younger rats, renal cells from aged rats displayed a decreased sensitivity to PTH. Forskolin-stimulated adenylyl cyclase activity did not decrease with age, but CTX-stimulated adenylyl cyclase did, suggesting an involvement of G proteins in age-related desensitization. Interestingly, this phenomenon did not occur in parathyroidectomized rats, suggesting that it was an adaptive response to the increased PTH levels that normally occur with aging in both humans (24) and rats (3). In contrast to our results with osteoblasts, Sacktor et al. (26) reported an age-related decrease in PTH receptor number in rat renal cell membranes. Clearly, a decrease in PTH receptor number combined with a defect in the adenylyl cyclase-G protein complex could contribute to the age-related decreased sensitivity of rat renal cells to PTH. A similar decreased sensitivity of adenylyl cyclase in osteoblast-like cells preexposed to PTH has been reported (5). Our data suggest a decreased sensitivity to PTH may also be occurring in bone cells from aged rats. Thus, as rats age, PTH levels increase, resulting in a decreased sensitivity to PTH such that osteoblastic cells are less responsive to subsequent exposure to PTH. However, this decreased sensitivity differs from that reported in aged renal cells in that PTH binding kinetics do not change.

The consequences of decreased osteoblastic sensitivity to PTH were not examined in this study. However, we found that the decreased responsiveness to PTH occurs by the 12th mo of age. Interestingly, Roholl et al. (25) found that trabecular bone volume as a percentage of tissue volume (BV/TV) in rats peaks at ~4 mo and then declines through 12 mo of age, reaching the lowest values at 20–25 mo of age. The values of BV/TV at 4–5, 12, and 20–25 mo reported by Roholl et al. closely parallel the PTH-stimulated cAMP accumulation we observed in osteoblasts from 4-, 12-, and 24- to 28-mo-old rats. It is tempting to speculate that changes in bone volume in rats as a function of age may partly be explained by decreased sensitivity of osteoblasts to anabolic effects of PTH.

In summary, our results suggest that osteoblastic cells isolated from 12- and 24- to 28-mo-old rats display an attenuated cAMP response to PTH. Furthermore, the mechanism underlying this decreased sensitivity appears to involve an uncoupling of Gs protein to adenylyl cyclase.

We thank Debbie Firestone for help in preparing this manuscript and Amy Kosh and Chris Beecher for technical assistance.

This work was supported by Grant AG-10199 from the National Institute of Aging (to H. J. Donahue) and DK-46919 from the National Institute for Diabetes, Digestive and Kidney Disease (to L. K. McCauley).

Some of the work presented in this manuscript was presented at the Eighteenth Annual Meeting of the American Society of Bone and Mineral Research held in Seattle, WA, in 1986.

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Received 15 May 1997; accepted in final form 3 July 1997.

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