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Characterization of PTH/PTHrP Receptor in Rat Duodenum: Effects of Ageing

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Abstract In rat enterocytes, signaling through the paratyroid hormone (PTH)/PTH-related peptide receptor type 1(PTHR1) includes stimulation of adenylyl cyclase, increases of intracellular calcium, activation of phospholipase C, and the MAP kinase pathway, mechanisms that suffer alterations with ageing. The purpose of this study was to evaluate whether an alteration at the level of the PTH receptor (PTHR1) is the basis for impaired PTH signaling in aged rat enterocytes. Western Blot analysis with a specific monoclonal anti-PTHR1 antibody revealed that a 85 kDa PTH binding component, the size expected for the mature PTH/PTHrP receptor, localizes in the basolateral (BLM) and brush border (BBM) membranes of the enterocyte, being the protein expression about 7-fold higher in the BLM. Two other bands of 105 kDa (corresponding to highly glycosylated, incompletely processed receptor form) and 65 kDa (proteolytic fragment) were also seen. BLM PTHR1 protein expression significantly decreases with ageing, while no substantial decrease was observed in the BBM from old rats. PTHR1 immunoreactivity was also present in the nucleus where PTHR1 protein levels were similar in enterocytes from young and aged rats. Immunohistochemical analysis of rat duodenal sections showed localization of PTHR1 in epithelial cells all along the villus with intense staining of BBM, BLM, and cytoplasm. The nuclei of these cells were reactive to the PTHR1 antiserum, but not all cells showed the same nuclear staining. The receptor was also detected in the mucosae lamina propria cells, but was absent in globets cells from epithelia. In aged rats, PTHR1 immunoreactivity was diffused in both membranes and cytoplasm and again, PTH receptor expression was lower than in young animals, while the cell nuclei showed a similar staining pattern than in young rats. Ligand binding to PTHR1 was performed in purified BLM. rPTH(1-34) displaced [I^{125}]PTH(1-34) binding to PTHR1 in a concentration-dependent fashion. In both, aged (24 months) and young (3 months) rats, binding of [I^{125}]PTH was characterized by a single class of high-affinity binding sites. The affinity of the receptor for PTH was not affected by age. The maximum number of specific PTHR1 binding sites was decreased by 30% in old animals. The results of this study suggest that age-related declines in PTH regulation of signal transduction pathways in rat enterocytes may be due, in part, to the loss of hormone receptors. J. Cell. Biochem. 88: 1157–1167, 2003.

Key words: PTH; PTH receptor; rat enterocytes; ageing

INTRODUCTION

Parathyroid hormone (PTH) is an 84-amino-acid polypeptide hormone functioning as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis [Strewler, 2000].

Very small decrements in serum calcium levels induce the secretion of PTH from the parathyroid glands initiating a rapid response to raise serum calcium levels by acting directly on kidney and bone or indirectly on intestine (via 1,25(OH_2) vitamin D_3) facilitating calcium absorption [Potts et al., 1997; Silverberg et al., 1999]. Signaling through the parathyroid hormone (PTH)/PTH-related peptide receptor (PTHR) involves the activation of two major transduction systems, one involving adenylyl cyclase [Partridge et al., 1981] and another involving phospholipase C [Civitelli et al., 1988]. Studies in vitro and in vivo have shown that the N-terminal 1-34 synthetic fragment of the 84 amino acids of PTH are necessary and

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sufficient for full biological activity of the intact hormone [Habener et al., 1984]. Moreover, requirement of the first two amino acids in the activation of adenylyl cyclase [Fujimori et al., 1991] and residues 29-32 in the activation of PLC stimulation [Jouishomme et al., 1994] and residues 25-34 as the principal receptor-binding region [Gardella et al., 1993] have been demonstrated. Recently, PTH was shown to activate the mitogen activated protein kinases (MAPKs) in osteoblastic cells [Swarthout et al., 2001], Chinese hamster ovary cells [Verheijen and Defize, 1997], and kidney cells [Cole, 1999; Lederer et al., 2000]. This activation is associated with increases in proliferation in osteoblastic and kidney cells [Cole, 1999; Swarthout et al., 2001]. In rat duodenal cells (enterocytes), we have previously reported the ability of PTH (1-34) to activate adenylyl cyclase and protein kinase A [Picotto et al., 1997], Gq-mediated activation of PLC and ultimately PKC [Massheimer et al., 2000], release of Ca^{2+} from intracellular stores and Ca^{2+} influx through voltage-dependent calcium channels [Gentili et al., in press]. Activation of PTH receptor in enterocytes, also leads to tyrosine phosphorylation of a number of intracellular proteins, the most prominent being PLCγ [Gentili and de Boland, 2001a; Gentili et al., 2001b] and the mitogen-activated protein kinases ERK1 and ERK2, which leads to an increase in DNA synthesis [Gentili and de Boland, 2000].

In most cells, PTH initiates its effects by interacting with the heterotrimeric G-protein-coupled PTH/PTHrP receptor type 1 (PTHR1), characterized by seven transmembrane domains, a long amino-terminal extracellular domain, and a long carboxyl-terminal intracellular tail [Abou-Samra et al., 1992]. To date, there appears to be a single PTH receptor found on osteoblasts, the PTHR1 [Juppner, 1999], although a second PTH receptor (PTHR2) has been described [Usdin et al., 1995, 1996] and others have been postulated [Orloff and Stewart, 1995]. However, the expression of the PTHR2 appears to be limited to pancreas, brain, kidney, and testis and this isoform does not bind PTHrP [Juppner, 1999]. Recently, three receptors for PTH/PTHrP have been isolated from zebrafish (Danio rerio) [Rubin and Juppner, 1999; Rubin et al., 1999]. Two of these receptors are homologues of PTHR1 and PTHR2, while the third (PTHR3) appears to be novel and has not been identified in mammalian cells to date [Juppner, 1999; Rubin et al., 1999]. Type 1 PTHR is distributed in several rat tissues, and the presence of PTHR1 protein and its mRNA in the small intestine, has been demonstrated by immunohistochemistry and in situ hybridization studies [Watson et al., 2000a]. However, a complete characterization and binding properties of rat enterocytes PTHR1 with ageing has not been reported. In the present study, we examined the localization, expression, and binding properties of the PTH/PTHrP type 1 receptor in enterocytes isolated from 3 and 24 month old rats.

MATERIALS AND METHODS

Chemicals

Synthetic rat PTH (1-34), leupeptin, aprotinin, Immobilon P (Polyvinylidene difluoride, PVDF) membranes were from Sigma Chemical Co. (St. Louis, MO). [^{125}I]-[Nie8, 21, Tyr34]-Parathyroid Hormone1-34 Amide (rat) was from New England Nuclear (Chicago, IL). Mouse monoclonal antibody against PTH receptor opossum kidney was from BABCO (Richmond, CA), rabbit anti-lamin B and rabbit anti-Gαs antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) were obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were of analytical grade.

Animals

Male Wistar rats (3 and 24 month-old) were fed with standard rat food (1.2% Ca; 1.0% phosphorus), given water ad libitum and maintained on a 12 h light-12 h dark cycle. Animals were sacrificed by cervical dislocation.

Isolation of Duodenal Cells

Duodenal cells were isolated essentially as previously described [Massheimer et al., 1994]. The method employed yields preparations that contain only highly absorptive epithelial cells and are devoid of cells from the upper villus or crypt [Weiser, 1973; Walters and Weiser, 1987] The duodenum was excised (10 cm starting from the pylorus), washed, and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A: 96 mM NaCl, 1.5 mM KCl, 8 mM

KH2PO4, 5.6 mM Na_2HPO_4, 27 mM Na citrate, pH 7.3, for 10 min at 37°C. The solution was discarded and replaced with solution B (isolation medium): 154 mM NaCl, 10 mM NaH_2PO_4, 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 5.6 mM glucose, pH 7.3, for 15 min at 37°C with vigorous shaking. The cells were sedimented by centrifugation at $155 \times g$ for 10 min, washed twice with solution C: 154 mM NaCl, 10 mM NaH_2PO_4, 5.6 mM glucose, pH:7.4 and resuspended in the incubation medium (solution D): 154 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM NaMOPS pH 7.4, 5.6 mM glucose, 0.5% BSA, 1 mM $CaCl_2$. All the above steps were performed under a 95% O_2–5% CO_2 atmosphere and using oxygenated solutions. The enterocytes were used between 20 and 60 min after their isolation. Cell viability was assessed by trypan blue exclusion in dispersed cell preparations; 85–90% of the cells were viable for at least 150 min. Phase-contrast microscopy of preparations revealed no morphological differences between enterocytes isolated from young and old rats as in previous studies [Massheimer et al., 1999].

Subcellular Fractionation

Brush border membrane isolation. Purified brush border membrane (BBM) preparations were obtained essentially as previously described [Max et al., 1978]. The duodenum from four rats was excised, washed, and trimmed of adhering tissue. The intestine was slit lengthwise and the mucosa scraped with a Teflon-coated spatula. The scrapings from all animals were collected, mixed, and transferred to a beaker containing ice-cold buffer (2.5 mM sodium EGTA, 2 mM sodium HEPES, pH 7.4) The scrapings were suspended by suction into a syringe and then homogenized in a glass-Teflon-hand homogenizer (6 stokes). The homogenate was centrifuged in a Sigma 3K30 refrigerated centrifuge at $400 \times g$ for 20 min, 4°C. The supernatant was carefully removed. The pellet was then resuspended in 10 ml ice-cold buffer (5 mM $MgCl_2$ in the EGTA–HEPES buffer described above, pH 7.4) and layered on a gradient composed of 10 ml 50% sucrose in $MgCl_2$–EGTA–HEPES buffer and 10 ml of 60% sucrose. The gradient was centrifuged at $90{,}000 \times g$ for 75 min, 4°C (Beckman centrifuge, SW 50.1 rotor). Material at the lower interface, containing the brush borders was suspended in buffer and collected by centrifugation in at $27.000 \times g$ for 15 min, 4°C. This fraction was composed almost entirely of intact brush borders from intestinal mucosal cells. The resulting pellets were suspended in $MgCl_2$–EGTA–HEPES buffer and then homogenized in a glass–Teflon-hand homogenizer (10 stokes). The protein content of the BBM was measured according to Lowry [Lowry et al., 1951]. Cell membranes were stored at −80°C until use.

Basolateral membrane isolation. Purified basolateral membrane (BLM) preparations were obtained by a method previously reported with some modifications [Suzuki et al., 1989; Kim et al., 1996]. The duodenal cells were collected in buffer A (10 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 5 mM dithiothreitol) containing protease inhibitors (1 mM PMSF, 8 μg/ml leupeptin, and 8 μg/ml aprotinin). The cell suspension was homogenized in a Teflon-glass hand homogenizer (40 stokes) and centrifuged at $1000 \times g$ for 10 min, 4°C (Sorvall centrifuge, SM-24 rotor). The supernatant was centrifuged at $100{,}000 \times g$ for 30 min, 4°C (Beckman centrifuge, Ti 50 rotor). The resulting pellet was resuspended in 1.5 ml of 15% sucrose in buffer A and layered on a discontinuous sucrose density gradient composed of 2 ml of 30% sucrose in buffer A layered on 1.5 ml of 45% sucrose in buffer A. This gradient was centrifuged at $76{,}000 \times g$ for 3 h, 4°C (Beckman centrifuge, SW 50.1 rotor). The 15–30% (plasma membrane fraction) was collected, diluted in solution B (10 mM Tris-HCl, pH 7.4 containing protease inhibitors) and centrifuged at $100{,}000 \times g$ for 1 h, 4°C (Beckman centrifuge, Ti 50 rotor). The resulting pellets were suspended in assay buffer (20 mM HEPES, 100 mM NaCl, 1mM EDTA, 3 mM $MgSO_4$, pH 7.5 plus protease inhibitors as necessary) by suction into a syringe to uniformity. Protein concentration was determined according to Lowry [Lowry et al., 1951]. Cell membranes were stored at −80°C until use.

Nuclear fraction isolation. The nuclear fraction was obtained by a method previously reported with some modifications [Boland et al., 1991; Marcinkowska et al., 1997]. Enterocytes were homogenized in 10 ml of buffer A 10 mM Tris-HCl, pH 7.4, 1,6 M sucrose, 1 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na_3VO_3, 1 mM PMSF). The nuclei enriched fraction was collected after a 15-min centrifugation at $1000 \times g$ 4°C (Sigma 3K30 centrifuge), and layered on a sucrose cushion (2M). This gradient was centrifuged at $6000 \times g$ for 1 h 4°C. The pellet

(nuclei) was washed twice in buffer A and resuspended in 300 μl of buffer lysis (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1% nonidet P40, 0.25% Na deoxycholate with protease inhibitors: 1 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml aprotinin; and phosphatase inhibitors: 0.2 mM Na_3VO_3, 25 mM NaF) by suction into a syringe to uniformity. Protein concentration was determinated according to Lowry [Lowry et al., 1951] and nuclei were immediately frozen and stored at −80°C until used in the assays. In order to ensure the purity of the isolated fraction, preparations were stained with nuclear-specific colorants (DAPI, hydrochloric acetic orcein) and revealed a high yield of purified nuclei.

SDS–PAGE and Immunoblotting

Protein lysates (25 μg protein) dissolved in Laemmli sample buffer were separated on SDS–polyacrylamide (10%) gels [Laemmli, 1970] and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in TBS-T (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Tween-20 containing 5% dry milk). Monoclonal mouse anti-opossum PTHR, rabbit anti-lamin B, or rabbit anti-Gαs antibodies were diluted in TBS-T and were allowed to react with the membranes overnight at 4°C. Next, the membranes were washed three times in TBS-T, incubated with a 1:10,000 dilution of peroxidase-conjugated anti-rabbit secondary antibody or peroxidase-conjugated anti-mouse secondary antibody for 1 h at room temperature and washed three additional times with TBS-T. The membranes were then visualized using an enhanced chemiluminiscent technique (ECL, Amersham Corp.), according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA 94547) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

Immunohistochemistry (IHC)

IHC was performed essentially as described [Watson et al., 1995]. Rats were killed by CO_2 inhalation, and proximal duodenum was rapidly removed and fixed in ice-cold formol buffered or Bouin. Fixation was carried out overnight at 4°C after which the tissue was dehydrated with ethanol. Dehydrated tissue was embedded in paraffin and 4-μm sections cut and mounted on silanized slides. After retrieving fixation-concealed antigens, tissue sections were incubated in blocking solution (phosphate-buffered saline (PBS) containing bovine serum albumin (BSA)) and then incubated with primary antibody. The monoclonal mouse anti-opossum PTHR antibody obtained from BABCO was used at a 1:50 dilution in blocking solution and incubated for 45 min at room temperature. Following multiple PBS washes, the immune complexes were visualized using vectastain ABC peroxidase kit and DAB reagents according to the manufacturer's instructions. Controls were performed in which primary antibodies were replaced by non-immune serum. All sections were counterstained with hematoxylin before dehydration and mounting. Photomicrographs were taken with a Zeiss Axiolab microscope equipped with Zeiss MC 100 Spot.

Radioreceptor Assay

Binding assays were performed by as previously reported with some modifications [Hoare and Usdin, 1999]. Briefly, [^{125}I-Tyr^{34}]-rPTH(1-34) and increasing concentrations of unlabeled rPTH(1-34)(10^{-8}–10^{-12} M) were added to 300 μl of assay buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 3 mM $MgSO_4$, pH 7.5 plus protease inhibitors as necessary) containing 0.3% (w/v) non-fat dried milk, and 100 μM PMSF. Purified basolateral membranes (20 μl, 50 μg protein) were added to initiate the binding reaction. Following 90 min incubation at 21°C, the tubes were centrifuged at $14{,}000 \times g$ for 10 min at 4°C (Sigma 3K30, 12,154 rotor). The pellets were washed four times with assay buffer and then counted in a gamma counter. An aliquot of radioligand was counted to calculate the amount added to each assay tube. Non-specific binding was measured by including 10^{-6} M unlabeled rPTH(1-34) in the assay. Specific binding was calculated by subtracting radioligand binding in the presence of excess unlabeled rPTH(1-34) from the total binding. The dissociation constant (Kd) and maximal binding sites (B_{max}) were analyzed using Prism 3.0 (GraphPad Sofware Inc., San Diego CA).

Statistical Analysis

Statistical significance of the data was evaluated using Student's *t*-test [Snedecor and Cochran, 1967] and probability values below

0.05 ($P < 0.05$) were considered significant. Results are expressed as means ± SD from the indicated set of experiments.

RESULTS

The presence of the PTH/PTHrP type 1 receptor was examined in BBM and BLM fractions from rat duodenum by immunoblot analysis with a specific monoclonal anti-PTH receptor antibody. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) revealed in the BLM and BBM fractions the presence of an 85-kDa PTH immunoreactive protein (Fig. 1), the size expected for the mature PTH/PTHrP receptor [Nissenson et al., 1987; Shigeno et al., 1988; Kaufmann et al., 1994; Huang et al., 1995]. The protein expression of the 85 kDa receptor was 7-fold higher in the BLM than in the BBM. Two other bands of 105 kDa (perhaps corresponding to highly glycosylated, incompletely processed receptor form) and 65 kDa also were seen. Based on results in other systems [Karpf et al., 1990], it is likely that the 65-kDa PTH receptor form results from cleavage of the 85-kDa receptor by an endogenous proteolytic enzyme. The expression of BLM receptor protein significantly decreases with ageing (−3-fold, $P < 0.025$), while no substantial decrease was observed in the BBM from old rats. Cell

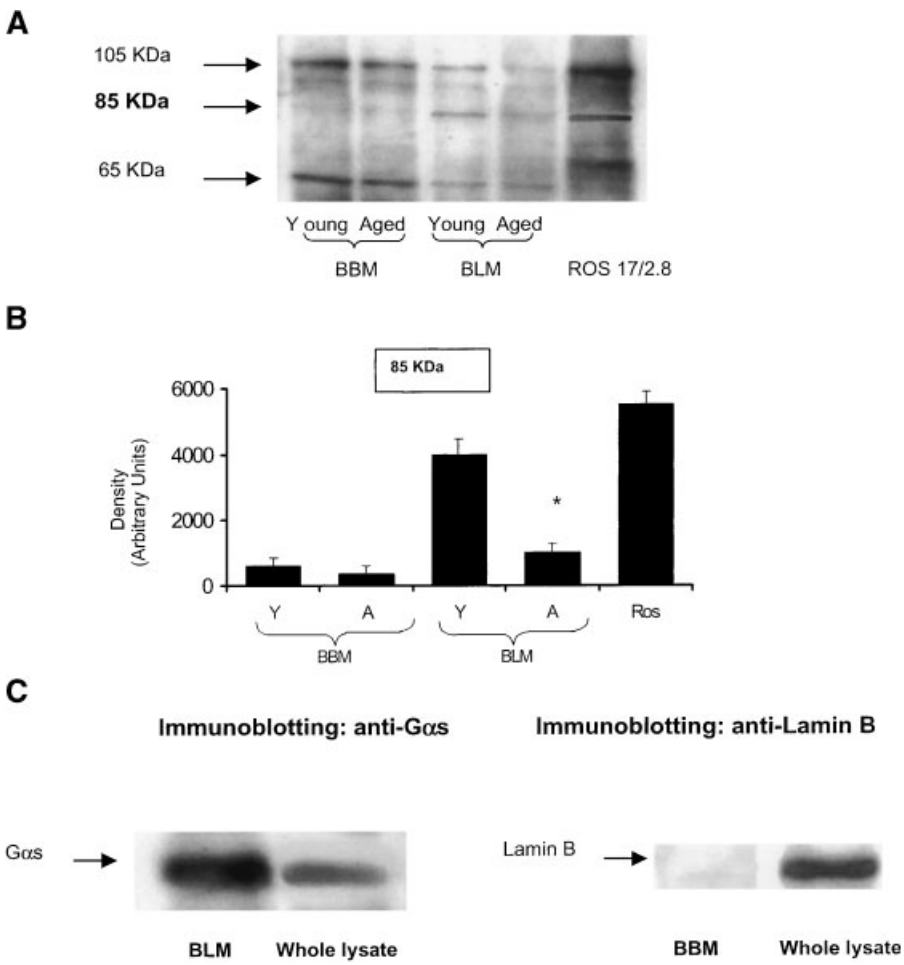

Fig. 1. PTH receptor levels in brush border membrane (BBM) and basolateral membrane (BLM) fractions from young (3 months) and aged (24 months) rats. Membrane proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting with anti-PTH receptor antibody as described under Methods. Cell lysates from the osteoblast-like osteosarcoma cell line (ROS 17/2.8) were used as positive control. **A**: Representative immunoblot. **B**: Bar graphs represent intensities of the 85 KDa PTHR1 quantified by scanning densitometry of blots from three independent experiments. *$P < 0.025$ respect to BLM from young rats. **C**: Proteins from membranes were immunoblotted with anti-Gαs BLM or anti-lamin B BBM antibodies. A representative immunoblot from three independent experiments is shown.

lysates from osteoblast-like osteosarcoma cell line (ROS 17/2.8) were used as a positive control in all the Western blot analysis, since the plasma membrane component of 80–85 kDa in these cells possesses the affinity, binding capacity, and physiological characteristics of the PTH receptor (PTHR1) [Shigeno et al., 1988]. As a further test of the purity of the isolated membranes, proteins from BLM and BBM were separated by SDS–PAGE, blotted and incubated with anti-Gαs or anti-lamin B antibodies. The BLM fraction was enriched in Gαs protein, a selective marker for plasma membrane, and the presence of lamin B, a nuclear-specific protein, was not detected in purified BBM (Fig. 1C).

Previous investigations using immunohistochemistry have reported that PTH receptor was present in the villus epithelium, intestinal crypt cells, and in interstitial cells, with each of these cell types exhibiting some nuclear staining [Watson et al., 2000a]. Therefore, we evaluate the presence of the PTH receptor in nuclei isolated of enterocytes from young and aged rats by Western blot analysis. As shown in Figure 2A, two bands of 65 and 105 kDa and a prominent 55-kDa proteolytic fragment were recognized by the anti-PTH receptor antibody, and the protein expression of PTH receptor was the same in the nuclei of young and aged animals. The purity of nuclear fraction was assessed by assaying for proteins, known to be associated with cellular components. Thus, lamin B immunoreactivity was determinated by Western blotting. Figure 2B shows the presence of this protein in cell nuclei and no detectable contamination of nucleus fragments was observed in the supernatant, as indicated by the absence of lamin B.

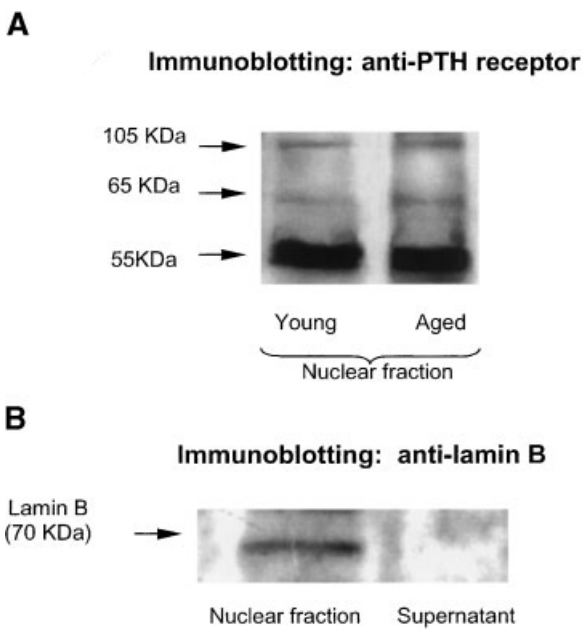

Fig. 2. PTH receptor levels in enterocytes nuclear fraction from young (3 months) and aged (24 months) rats. The cells nuclei were isolated and nuclei proteins were electrophoresed, electroblotted to PVDF membranes and incubated with anti-PTH receptor antibody as described in Methods. **A**: Representative Western blot of PTH receptor levels from three independent experiments. **B**: Representative Western blot of nuclear proteins immunoblotted with anti-lamin B antibody.

We further assessed the expression and localization of PTH/PTHrP type 1 receptor in enterocytes from young and aged rats by immunohistochemistry. As shown in Figure 3C,E, immunoreactive PTH receptor in tissue sections was observed in duodenal epithelial cells from young rats all along the villus and the enterocytes exhibited intense staining of BBM, BLM, and cytoplasm. The nuclei of these cells were reactive to the PTHR1 antiserum, but not all cells showed the same nuclear staining. According with these observations, there is evidence demonstrating that not all cells of a particular type within a given tissue had nuclear PTHR1 and that the nuclear localization of the PTH/PTHrP receptor is associated with cell proliferation [Watson et al., 2000b]. The receptor was also detected in the mucosae lamina propria cells, but was absent in goblets cells from epithelia. In aged rats (Fig. 3D,F), PTHR1 immunoreactivity was diffused in both membranes and cytoplasm. The nuclei from these cells had a similar staining pattern as the enterocytes from young rats. Again, and in agreement with immunoblotting analysis, in aged rats, PTH receptor expression analyzed by immunohistochemistry was markedly lower than in young animals.

We then examined the binding of I^{125}-PTH in purified BLM isolated from young and aged rats.

Homologous displacement assays were performed for estimation of the binding constants. rPTH(1-34) displaced [I^{125}]rPTH(1-34) binding to PTH receptor in a concentration-dependent fashion (Fig. 4). In both, aged and young rats, binding of [I^{125}]PTH was characterized by a single class of high-affinity binding sites. No significative differences were found in PTH receptor binding affinity with ageing (Kd: 0.20 ± 0.018 and 0.15 ± 0.028 nM, for young and aged rats, respectively). The maximun number of

rPTH binding sites (B_{max}) was reduced by 30% in BLM membranes from aged rats (310 ± 32 and 217 ± 20 fmoles/mg protein for BLM isolated form young and aged rats, respectively).

DISCUSSION

The results of the present investigation provide information of the PTH/PTHrP type 1 receptor present in enterocytes isolated from

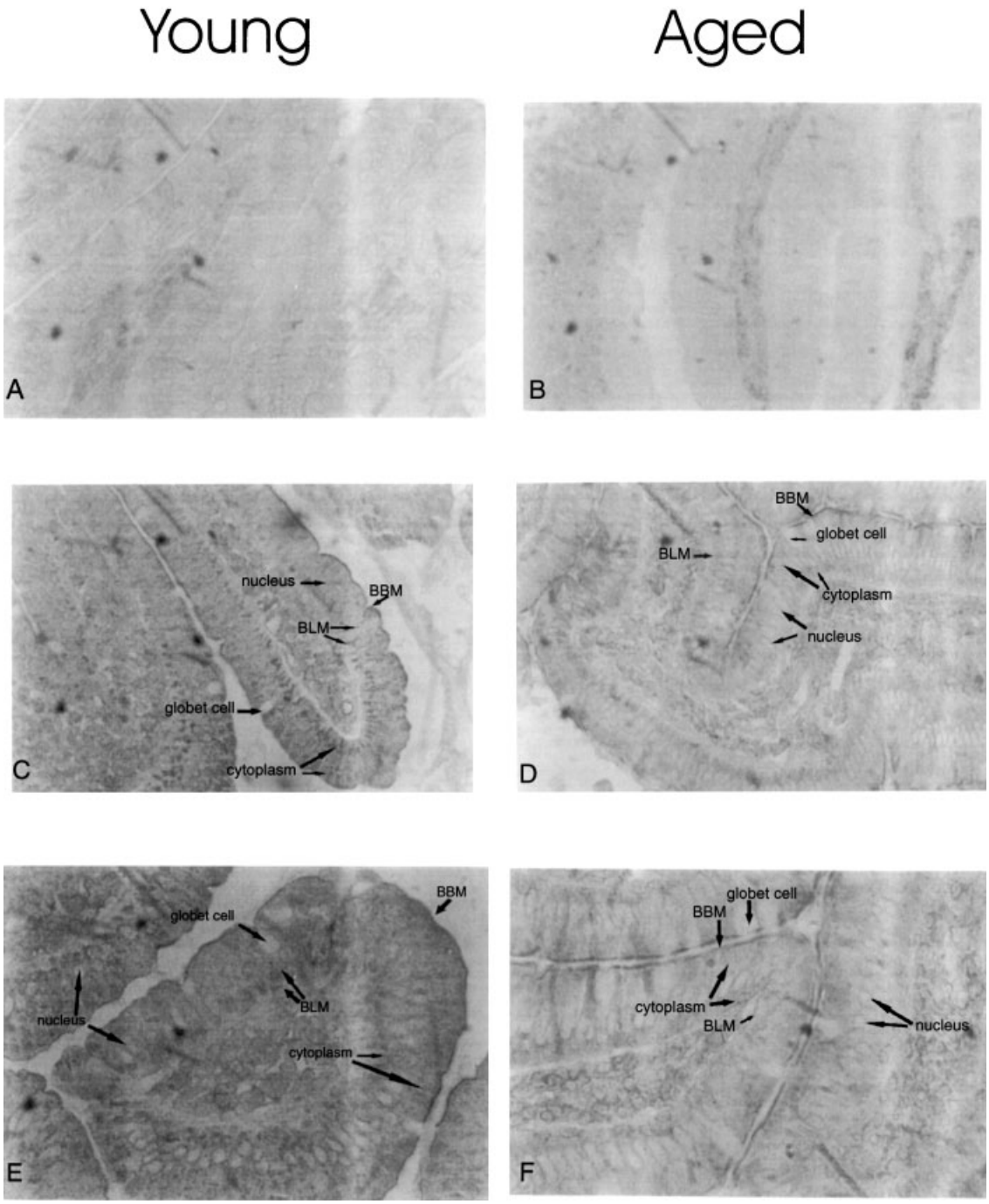

Fig. 3. Immunohistochemistry detection of PTH receptor in rat duodenum. Arrows indicate the localization of PTHR in BBM, BLM, and cytoplasm from young (**C** and **E**) and aged (**D** and **F**) enterocytes. The nuclei of these cells were reactive to the PTHR antiserum, but not all cells showed the same nuclear staining. The PTHR was also detected in cells from mucosae lamina propria. No staining of enterocytes was evident with normal serum instead of primary antibody in small intestine sections from young (**A**) and aged (**B**) rats. Original magnifications: A, B, C, and D, ×400; E and F, ×600.

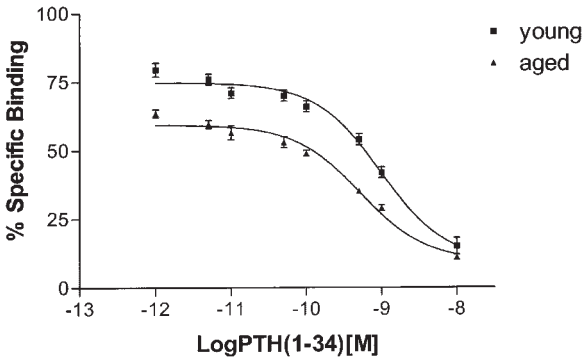

Fig. 4. Effect of increasing concentration of rPTH (1-34) on binding of ^{125}I-rPTH to BLM from young and aged rat enterocytes. Purified BLM were incubated with [^{125}I]rPTH (1.9×10^{-9} M) and various concentrations of unlabeled PTH and specific binding of hormone was determined as described in Methods. Radioligand binding data were analyzed by non-linear regression using Prism 3.0 (GraphPad Software Inc., San Diego, CA). For each experiment membranes were prepared from four rats from each age group. Data represent the mean ± SD of three independent experiments performed in triplicate.

3 and 24 month old rats and show that the protein expression of the receptor significantly decreases with ageing. The PTHR1, belongs to the type II family of G protein-linked receptors [Juppner et al., 1999] and can be divided into two functional domains, the large extracellular N-terminal domain has been proposed to provide most of the binding energy for receptor–ligand interaction, and the juxtamembrane region of the receptor is a determinant of receptor activation and second messenger generation [Bergwitz et al., 1996].

Previous studies reveal the widespread expression of PTH/PTHrP receptor gene in intestinal epithelial cells [Li et al., 1995] and confirm the broad distribution of PTHR1 in rat tissues [Watson et al., 2000a]. In agreement with these observations, here we show that PTHR1 is present in absorptive epithelial cells and another duodenal cell types and, in enterocytes from young and aged rats, the receptor was detected in the plasma membranes (BBM and BLM), cytoplasm, and nucleus.

In several systems, PTHR1 with full functional activity is an 80–85 kDa protein [Nissenson et al., 1987; Shigeno et al., 1988; Kaufmann et al., 1994]. In addition, recent investigations showed that purified human PTHR1 from COS-7 cells resolved by SDS–PAGE appears as a broad ~80 kDa band [Shimada et al., 2002]. According with these studies, immunoblot analysis revealed the presence of an 85 kDa protein, immunoreactive with PTHR1 antibody in the BLM, to a less extent in the BBM but this form is absent in the nucleus. Another PTH immunoreactive components were also seen in both membranes and in the nucleus, possible related to the fully processed receptor (105 kDa) and proteolytic fragments (65 and 55 kDa) and we couldn't exclude the possibility that these forms retain full functional activity.

The PTHR1 is the first member of GPCR to have a shown nuclear localization [Jans and Hassan, 1998]. It has been reported that PTHR1 is localized in the nucleus of some rat cells within kidney, liver, gut, uterus, and ovary. Furthermore, PTHR1 traffics to the nucleus during the time of DNA duplication in MC3T3-E1 cells when synchronized in culture [Watson et al., 2000b]. In line with these observations, our results indicated the presence of nuclear PTHR1 in enterocytes and reveals that the amount of basal protein expression determined by Western blot analysis was no different in the nucleus of duodenal cells from young and aged rats. Ligands such as those of the platelet derived and fibroblast growth factor classes, as well as cytokines or its receptors have been found to localize in the nucleus through the action of nuclear localization sequences [Jans and Hassan, 1998]. The significance of the PTHR1 in the nuclei of these cells remains to be determined, and although the signaling through the PTH receptor involves the activation of two major transduction systems, one involving adenylyl cyclase and another involving phospholipase C, direct nuclear effects cannot be ruled out. Perhaps, PTHR1 translocates to the nucleus to participate directly on gene regulation.

The presence of PTH receptor was also seen along the enterocyte cytoplasm from all rats by immunohistochemistry, but intense staining was observed between the nucleus and the basement membrane in absorptive cells from young rats. This observation could be explained due to the presence of the protein synthesis machinery nearer the nucleus [Stevens and Lowe, 1999] and is likely that the decreased staining observed in enterocytes from 24 month-old rats may be related to impair cellular activity with ageing.

Changes occur with age in the stem cells of murine small intestine crypts [Martin et al., 1998]. Regarded that the replication of the stem cells replenishes the stock of the other cells, including the Paneth and endocrine cells, its

main function is to replace the mucous cells and enterocytes of the villi since these cells have a rapid turnover. It is likely that any deterioration with age in the number and functional integrity of stem cells may have profound consequences for the maintenance of function and proliferative homeostasis in the tissues that rely upon these cells. Thus, in proximal small intestine from old Fisher rats, decreased survival of newly formed crypt epithelial cells was observed and the expression of several proteins in aged rats was markedly lower than in young animals [Holt et al., 1984]. In this regard, the development of intestinal BBM enzymes in Wistar rats was influenced by age during the postnatal period [Jang et al., 2000]. Moreover, the mRNA levels [Liang et al., 1994] and the number of duodenal 1,25-(OH_2)-vitamin D_3 receptors declined in the aged Wistar rats [Takamoto et al., 1990]. This data is consistent with our immunoblot and immunohistochemistry analysis showing that the BLM expression of the PTH receptor decreases with ageing.

The results reported in this study demonstrate that the dissociation constant of PTH from the receptor in isolated BLM is not affected by age. In contrast, the number of PTH binding sites is reduced in the aged rat. Agonist binding of hormone receptor requires both functional receptor and G proteins. The impairment of physiological responses to PTH in rat enterocytes from old rats is not limited to changes in PTH binding sites and receptor levels. Recently, we demonstrated that enterocytes from aged rats exhibited decreased levels of the α-subunits of both Gi and Gq/11 [Facchinetti and de Boland, 2001] as well as impaired activation of PI-PLC hydrolysis and adenylyl cyclase [Massheimer et al., 2000].

Ageing is known to alter many physiological processes, in fact many changes occur during cellular ageing, such as decreased membrane fluidity, increased protein oxidation, decreased DNA methylation, and defects in mitogenic signaling [Kirkland, 1992]. Ageing is associated with increased circulating PTH levels [Chan et al., 1992] and decreased serum vitamin D metabolites [Armbrecht et al., 1984], intestinal calcium absorption [Horst et al., 1978], and bone density [Hui et al., 1988]. Also, an age-related decline in PTH-stimulated AC activity in both rat kidney slices and cell membranes [Armbrecht et al., 1986; Hanai et al., 1989] has been shown. Upon ageing, reduced PTH stimulation of cAMP levels [Egrise et al., 1992] or an increase in hormone-dependent cAMP accumulation [Pfeilschifter et al., 1993] has been observed in bone cells. Ageing is known to alter many physiological processes within the intestine, including PTH-signaling. The hormone increased enterocyte $^{45}Ca^{2+}$ influx, the absolute levels of camp, and AC activity to a greater extent in aged than in young rats whereas the early production of IP_3 and DAG generated by PTH was blunted in old animals [Massheimer et al., 2000]. In enterocytes from aged rats, the hormone induced c-Src tyrosine dephosphorylation, a major mechanism of c-Src activation [Piwnica-Worms et al., 1987; Brown and Cooper, 1996], was also blunted and PLCγ phosphorylation via the non-receptor tyrosine kinase c-Src was impaired [Gentili et al., 2000b]. Although the relative levels of p42 and p44 MAPK did not change with age, the magnitude of PTH-dependent MAPK phosphorylation was significantly lower in enterocytes of aged rats compared with those of young animals [Gentili and de Boland, 2000].

A deficiency in PTH receptor and G-protein expression with ageing may have important consequences for correct receptor/effector coupling in the duodenal tissues and may explain age-related declines in the hormonal regulation of signal transduction pathways.

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