Effect of ageing in the early biochemical signals elicited by PTH in intestinal cells

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Abstract

In previous work, we have demonstrated that rPTH(1-34) increases cytoplasmic calcium concentration ([Ca²⁺]) in isolated rat enterocytes. In the present study, we have identified the sources of PTH-mediated increase in [Ca²⁺] and the implication of Ca²⁺ on hormone early signals in enterocytes isolated from young (3-month-old) and aged (24-month-old) rats. In young enterocytes, PTH raised [Ca²⁺] in a dose-dependent manner (1 pM–100 nM). In cells from aged rats, hormone concentrations higher than physiological (≥ 1 nM) were required to observe significant increases in [Ca²⁺]. Phospholipase C (PLC) inhibitors blocked the initial acute elevation of the [Ca²⁺], biphasic response to PTH of young enterocytes while in old cells, no effects were observed. The voltage-dependent calcium-channel blocker (VDCC), nitrendipine, suppressed PTH-dependent changes of the sustained [Ca²⁺] phase in young and aged animals. In this study, we analysed, for the first time, alterations in phosphatidylinositol 3-kinase (PI3K) activity and response to PTH in rat enterocytes with ageing. Basal PI3K activity was significantly modified by ageing. Acute treatment with 10⁻⁵ M PTH increased enzyme activity, with a maximum at 2 min (+3-fold) in young rats and only elevated by less than 1-fold basal PI3K activity in aged animals. Hormone-induced tyrosine phosphorylation of p85α, the regulatory subunit of PI3K, as well as the phosphorylation on Thr308 of its downstream effector Akt/PKB was evident in enterocytes from 3-month-old rats, whereas it was greatly reduced in the cells from 24-month-old animals. Intracellular Ca²⁺ chelation (BAPTA-AM, 5 μM) affected the tyrosine phosphorylation of p85α and inhibited PTH-dependent PI3K activation by 75% in young rats and completely abolished the enzyme activity in aged animals, demonstrating that Ca²⁺ is required for full activation of PI3K in enterocytes stimulated with PTH. The Thr phosphorylation of PI3K downeffector, Akt/PKB, was also fully dependent on Ca²⁺. Taken together, these results suggest that PTH regulation of enterocyte [Ca²⁺], involves Ca²⁺ mobilization from IP₃-sensitive stores and the influx of the cation from the extracellular milieu, the former pathway being blunted during ageing. The data also indicates a positive role for intracellular calcium in one of the early signals of PTH in rat enterocytes, the activation of PI3K, and that hormone regulation of PI3K activity and Akt/PKB phosphorylation on Thr308 is impaired with ageing.

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Keywords: Parathyroid hormone; Intracellular Ca²⁺; PI3K; AKT/PKB; Intestine; Ageing

1. Introduction

Parathyroid hormone (PTH) is an essential regulator of calcium homeostasis [1]. PTH stimulates both adenyl cyclase (AC) [2] and phospholipase C (PLC) [3] as a result of ligand binding to the heterotrimeric G-protein-coupled PTH/PTHrP receptor [4,5]. The ability of PTH to activate adenyl cyclase and protein kinase A (PKA) is mediated via the stimulatory GTP-binding protein (Gs). PTH also directs the Gq-mediated activation of PLC and ultimately PKC [3], release of calcium from intracellular stores [6], and activation of calcium channels [7,8]. PTH stimulation of other signaling pathways involving PLA₂ [9] or PLD [10] has also been described though the details are less well characterized. Emerging evidence indicates that PTH can regulate mitogen-activated protein kinases (MAPK) activity in a cell-specific and G protein type-dependent manner [11]. However, the role of G-protein-coupled receptors and their ligands in intestinal epithelial cell signalling remains poorly understood. More recently, we have shown that PTH increases rat duodenal cells calcium influx, which involve the stimulation of dihydropyridine-sensitive calcium chan-
nels and activation of the AC/cAMP and PLC/IP3/DAG second messenger pathways [12,13]. Furthermore, PTH rapidly, and in a dose-dependent fashion, stimulates Src tyrosine kinase activity and consequently downstream the tyrosine phosphorylation of several enterocyte proteins [14], among which two major targets of the hormone could be immunochemically identified as PLCγ and the growth-related protein MAPK (p42/44-MAPK), also known as extracellular signal-regulated kinases (ERK1/2) [15,16]. In addition, recent evidence shows that cAMP and Ca2+ play a role upstream in the signalling mechanism leading to PTH activation of MAPK in these cells [17]. Of physiological significance, in agreement with the mitogenic role of the MAPK cascade, PTH increased enterocyte DNA synthesis [17].

Many changes occur during cellular ageing, such as decreased membrane fluidity, increased protein oxidation, decreased DNA methylation and defects in mitogenic signalling [18]. Ageing is associated with increased circulating PTH levels [19] and decreased serum vitamin D metabolites [20], intestinal calcium absorption [21] and bone density [22]. Also, an age-related decline in PTH-stimulated AC activity in both rat kidney slices and cell membranes [23,24] has been shown. Upon ageing, reduced PTH stimulation of cAMP levels [25,26] or an increase in hormone-dependent cAMP accumulation [27] has been observed in bone cells. In rat duodenal cells, we have obtained evidence of alterations in PTH-signaling systems with ageing. The hormone increased enterocyte 45Ca2+ influx, the absolute levels of cAMP and AC activity to a greater extent in aged than in young rats [13] whereas the early production of IP3 and DAG generated by PTH was blunted in old animals. In enterocytes from aged rats, the hormone-induced c-Src tyrosine dephosphorylation, a major mechanism of c-Src activation [28,29], was also blunted and PLCγ phosphorylation via the nonreceptor tyrosine kinase c-Src was impaired [15]. 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 [16].

The present study was undertaken to identify the sources of PTH-mediated increase in [Ca2+], the implication of Ca2+ on hormone-dependent PI3-kinase activation and to further examine the influence of ageing in these early signals elicited by PTH in rat duodenal cells.

2. Materials and methods

2.1. Materials

Synthetic rat PTH (1–34), fura 2/AM, thapsigargin, neomycin, Immobilon P (polyvinylidene difluoride, PVDF) membranes and phosphatidylinositol were from Sigma Chemical Co. (St. Louis, MO, USA). Bayer (Leverkusen, Germany) supplied nitrendipine. Compounds U-73122 [1-(6-

2.2. Animals

Young (3–6-month-old) and aged (20–24-month-old) male Wistar rats were fed with standard rat food (1.2% calcium; 1.0% phosphorous), given water ad libitum and maintained on a 12 h light–12 h dark cycle. Animals were killed by cervical dislocation.

2.3. Duodenal cell isolation

Duodenal cells were isolated as described previously [30]. The method employed yields preparations containing only highly absorptive epithelial cells that are devoid of cells from the upper villus or crypt [31]. The duodenum was excised, washed with 0.9% NaCl and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A containing (in mM): 96 NaCl, 1.5 KCl, 8 KH2PO4, 5.6 Na2HPO4, 27 Na citrate, pH 7.3, for 10 min at 37 °C. The solution was discarded and replaced with isolation medium containing (in mM): 154 NaCl, 10 NaH2PO4, 1.5 EDTA, 0.5 diethiothreitol, 5.6 glucose, pH 7.3, for 15 min at 37 °C with continuous shaking (87 oscillations/min). The cells were sedimented by centrifugation at 750 × g for 10 min, washed twice with 154 mM NaCl, 10 mM NaH2PO4, 5.6 mM glucose, pH 7.3, and then resuspended in measurement buffer (see below). All the steps mentioned above were performed under an atmosphere of 95% O2/5% CO2. Cell viability was assessed by the Trypan Blue technique. Exclusion of the dye in >90% of the cells was observed for at least 90 min after isolation. Morphological characterization was performed by phase-contrast microscopy. Enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cells [31].

2.4. Measurement of intracellular calcium [Ca2+]

Enterocytes were loaded with the fluorescence calcium indicator fura 2/AM for 10 min at 37 °C in the dark, in
Fura 2-loaded enterocytes from young (3 months) and aged (24 months) rats were preincubated in the presence or absence of U-73122 (20.5 mM) for 3 min and then exposed to 10 nM PTH. Intracellular calcium concentration ([Ca2+]i) was measured as described in Materials and methods. Data are expressed as means of seven independent recordings for young and aged cells, respectively, and correspond to the value of [Ca2+]i at the sustained phase (5 min) of PTH response. *P < 0.001.

Loading buffer containing (in mM) 138 NaCl, 5 KCl, 1 MgCl2, 5 glucose, 10 HEPES [32], pH = 7.4, 1.5 CaCl2, 0.1% bovine serum albumin, 0.012% pluronic F127 and 4 µM fura 2/AM. The cell suspension was washed with measurement buffer containing (in mM) 138 NaCl, 5 KCl, 1 MgCl2, 5 glucose, 10 HEPES, pH = 7.4, 1.5 CaCl2, in order to eliminate unloaded dye, and maintained at room temperature for 15 min in the dark until used. For measurements of intracellular fura 2 fluorescence signals, cells were placed into quartz cuvettes at a density of 10⁶/ml and then introduced into the sample compartment of a thermostatically controlled (37 °C) SLM Aminco 8100 spectrofluorimeter (Spectronics Inc., MO, USA) under constant low-speed stirring. Hormone and inhibitors were added directly into the cuvette at the indicated times. Water was used as vehicle for PTH (1–34) and verapamil while U73122, nitrendipine and neomycin were dissolved in ethanol (<0.1%) and thapsigargin in dimethylsulfoxide (<0.01%). Fura 2-fluorescence intensity was monitored at an emission wavelength of 500 nm by alternating the excitation wavelength between 340 and 380 nm with a dual excitation monochromator (DMX 1100). Signals were ratioed (R = 340:380) and maximal (Rmax) and minimal (Rmin) intracellular dye fluorescence were determined by adding Triton X-100 (0.1% final concentration) and EGTA (5 mM, pH>8.3), respectively. This procedure ensures that the plateau phase of the recorded changes is not due to massive release of fura into the medium. Transformation of fluorescence signals into [Ca2+]i was performed according to Grynkiewicz et al. [33]. The use of Triton X-100 in the calibration procedure did not interfere with the fluorescence signal of fura 2. Under the present conditions of measurement, the dissociation constant (Kd) for the fura 2/Ca2+ complex was assumed to be 224 nM [34]. All saline solutions used were prepared with deionized water.

### Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>[Ca2+]i (nM)</th>
<th>Young</th>
<th>+ U-73122</th>
<th>+ Neomycin</th>
<th>Aged</th>
<th>+ U-73122</th>
<th>+ Neomycin</th>
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<td>1</td>
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<td>134 ± 13</td>
<td>135 ± 12</td>
<td>158 ± 12</td>
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<tr>
<td>2</td>
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<td>162 ± 14*</td>
<td>159 ± 16*</td>
<td>161 ± 14</td>
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<tr>
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<tr>
<td>5</td>
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<td>246 ± 12*</td>
<td>248 ± 16*</td>
<td>277 ± 16*</td>
<td>281 ± 23*</td>
<td>284 ± 21*</td>
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Fura 2-loaded enterocytes from young (3 months) and aged (24 months) rats were preincubated in the presence or absence of U-73122 (2 µM) or neomycin (0.5 mM) for 3 min and then exposed to 10 nM PTH. Intracellular calcium concentration ([Ca2+]i) was measured as described in Materials and methods. Data are expressed as means of seven independent recordings ± S.D. and correspond to the [Ca2+]i at each indicated time. *P < 0.001, with respect to the control (time 0), incubated with or without compound U-73122 or neomycin in the absence of PTH.

### 2.5. Immunoprecipitation

Enterocytes were treated with PTH and then lysed. Lysate aliquots (500–700 µg protein) were incubated overnight at 4 °C with the corresponding primary antibodies, followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed three times with cold immunoprecipitation buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM sodium orthovanadate, 1% Triton X-100 and 1% NP40), two times with PBS and then subjected to Western blot analysis.

### 2.6. Western blot analysis

Proteins were separated by one-dimensional SDS-PAGE [35]. Briefly, samples were mixed with 2 × Laemmli sample buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol and 0.02% bromophenol
blue) and heated for 5 min at 95 °C. Proteins (25 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide minigels and then transferred to Immobilon P (PVDF) membranes. The membranes were immersed in TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 5% skim milk for 2 h to block nonspecific binding. Anti-PI3K (p85α), anti-phospho Akt/PKB (Thr308) or antiphosphotyrosine antibodies were allowed to react with the membrane overnight at 4 °C. The membranes were then twice washed (5 min) with TBS-T (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), followed by one 10-min wash with TBS-T. The membranes were incubated with 1 μg/ml of peroxidase-labeled goat anti-mouse IgG antibody in TBS-T for 1 h at room temperature. After two washes with TBS-T, the membrane was visualized by using an enhanced chemiluminiscent technique (ECL), according to the manufacturer’s instructions. Images were obtained with a model GS-700 Imaging Densitometer from Bio-Rad (Hercules, CA 94547, USA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

2.7. PI3K assay

PI3K were immunoprecipitated from control or PTH-stimulated cells (700 μg protein) using anti-phosphotyrosine. The immunoprecipitates were washed three times with ice-cold lysis buffer and then incubated in 50 μl kinase buffer (10 Mn Tris–HCl pH 7.4, 150 Mm NaCl, 5 mM EDTA, 0.1 Mm Na ortovanadate) containing 0.2 mg/ml phosphatidylinositol (PI) at 37 °C for 10 min. The assay was initiated by adding 50 μCi [γ32P]ATP (10 Ci/mmol) and 20 mM MgCl2, and terminated by adding 6 N HCl (20 μl), and the phosphoinositol lipids were extracted with chloroform/methanol (2:1). The phospholipid contained in the organic phase were recovered, dried, resuspended in chloroform, spotted on a silica gel 60 thin layer chromatography plates and separated in chloroform/methanol/28% ammonium hydroxide/water (120:94:4:22.6). The phosphorylated products were visualized by autoradiography and quantified using the Molecular Analyst program (Bio-Rad).

2.8. Statistical evaluation

Statistical significance of data was evaluated using Student’s t-test [36] and probability values below 0.050 (P<0.050) were considered significant. In addition, for multiple comparisons, Bonferroni test and analysis of variance were employed. Quantitative data are expressed as the means ± S.D. from the indicated set of experiments.

3. Results

Recent studies have shown that PTH induces changes in cytoplasmic Ca2+ levels in rat enterocytes characterized by an almost immediate rise in [Ca2+]i (within 30 s), rapidly reaching a peak level, followed by a long-lasting plateau phase and that the early Ca2+ rise is absent in aged animals [13]. In the present study, it is shown that PTH increases intracellular calcium levels in enterocytes in a dose-dependent manner (Fig. 1). In young rats, the PTH-induced increments in [Ca2+]i at the plateau phase (5 min) were clearly evident within the physiological concentration range of the hormone (1–100 pM), the response rising until 10 nM where major differences with respect to basal [Ca2+]i (138 ± 10 nM) were reached (+84%). In cells from aged animals, higher PTH concentrations (1–100 nM) were required to observe significant changes in cytosolic Ca2+ above basal values (162 ± 14 nM). The next set of experiments was designed to identify the possible sources of PTH-mediated increase in [Ca2+]i in enterocytes. The mobilization of Ca2+ from intracellular stores leading to a rapid
increase in $[\text{Ca}^{2+}]_{\text{i}}$ is one of the earliest events stimulated by the binding of agonists to G-coupled protein receptors that signal through $G_{q}$-mediated activation of PLC [37]. In order to evaluate the participation of phosphoinositide hydrolysis in PTH response, we tested whether specific PLC inhibitors could block the increase in $[\text{Ca}^{2+}]_{\text{i}}$ induced by the hormone in rat duodenal cells. As shown in Table 1, compound U-73122 (2 $\mu$M), an aminosteroid which has a selective inhibitory effect on PLC-mediated biological activities [38], blocks the immediate rise in $[\text{Ca}^{2+}]_{\text{i}}$ when cells from young rats were exposed to PTH, while the sustained phase (4–5 min) was not altered, thus indicating that the initial phase of the response to PTH of young cells is mediated by $\text{Ca}^{2+}$ mobilization from intracellular stores. The selectivity of U-73122 action was evidenced by the observation that compound U-73343, an inactive analog of U-73122 [38], was not able to block hormone-induced effects on intestinal $[\text{Ca}^{2+}]_{\text{i}}$ (not shown). In agreement with the absence of PLC activation and IP$_3$ release previously reported [13], the $[\text{Ca}^{2+}]_{\text{i}}$ response induced by PTH in aged duodenal cells was not affected by preincubation with U73122 (Table 1). Similar results were obtained when the cells from young and aged animals were treated in the presence of 0.5 mM neomycin, an aminoglycoside antibiotic that binds poly-phosphoinositides making them unavailable to PLC [39]. We then examined the effects of $\text{Ca}^{2+}$-channel inhibition on hormone-induced increase of $[\text{Ca}^{2+}]_{\text{i}}$. As shown in Fig. 2 (upper panel), the voltage-dependent calcium channel blocker nitrendipine (1 $\mu$M) reduced by 75% the ability of PTH to increase the sustained phase of $[\text{Ca}^{2+}]_{\text{i}}$, in enterocytes from young animals while in old rats the calcium channel blocker completely suppressed the hormone response (Fig. 2, lower panel). The presence of fully competent intracellular calcium deposits in aged cells was evidenced by the immediate increase in cytoplasmic $\text{Ca}^{2+}$ observed after addition of 1 M thapsigargin, a sesquiterpene that selectively liberates stored $\text{Ca}^{2+}$ by inhibiting the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase [40].

Besides the generation of IP$_3$ [13], which induces the rapid release of $\text{Ca}^{2+}$ from internal stores, PTH triggers the activation of phosphatidylinositol-3’-kinase (PI3K) in rat enterocytes [41]. PI3K is one of the most important regulatory proteins involved in different signalling path-
ways and controlling many physiological processes, including regulation of cell growth, proliferation, survival and differentiation. In this study, we analyzed, for the first time, alterations in PI3K activity and response to PTH in rat enterocytes with ageing. To that end, enterocytes isolated from 3- and 24-month-old rats were briefly stimulated with $10^{-8}$ M PTH, dose selected from previous studies [41], and PI3-kinase activity was measured after immunoprecipitation of the cell lysates with anti-phosphotyrosine. Assays with phosphatidylinositol as a substrate revealed that basal PI3K activity is modified by ageing (+1-fold) (Fig. 3). Treatment with PTH increased enzyme activity in young rats, peaking at 2 min (+3-fold) but did not substantially modified basal activity in aged animals (less than 1-fold, 2 min). The role of calcium in the signalling cascade leading to increase PI3K activity by PTH in intestinal cells is not yet characterized. We then examined the effects of the intracellular Ca$^{2+}$ chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N,N$^\prime$-tetraacetic acid, sodium (BAPTA-AM, 5 $\mu$M) on hormone-induced PI3K activity. As presented above, an optimal increase in enterocyte PI3K activity was observed once the cells were stimulated with $10^{-8}$ M PTH for 2 min (Fig. 3 and Ref. [41]). We thus used these conditions to examine the effects of intracellular calcium chelation. As shown in Fig. 4, BAPTA-AM inhibited PTH-induced activation of PI3K by 85% in young enterocytes and suppressed PTH effects in cells from old animals. As shown in Fig. 5, incubation of young enterocytes with PTH increased the level of tyrosine phosphorylation of the regulatory p85$\alpha$ subunit of PI3K, peaked at 2 min (+3-fold) and reached values near basal after 15-min exposure, with similar kinetics to those found for the increase in kinase activity (Fig. 3). Although basal levels of p85$\alpha$ tyrosine phosphorylation were greatly higher in old enterocytes, hormone effects were diminished by 75%. We next evaluated the implication of Ca$^{2+}$ in PTH-induced tyrosine phosphorylation of the regulatory p85$\alpha$ subunit of PI3K. To that end, enterocytes were again stimulated for 2 min with $10^{-8}$ M PTH in the presence of BAPTA-AM (5 $\mu$M) or EGTA (0.5 mM). Chelation of intracellular calcium or its removal from the external medium clearly decreased PTH-dependent P85$\alpha$ tyrosine phosphorylation (Fig. 6).

An important downstream effector of PI3K is the serine-threonine kinase Akt, or protein kinase B (PKB). The effect
of ageing in the ability of PTH to stimulate the phosphorylation of Akt/PKB was therefore investigated. As shown in Fig. 7, treatment of enterocytes from 3-month-old rats with PTH led to the rapid (within 30 s) phosphorylation of Akt/PKB, as determined by immunoblot analysis with an antibody specific for Thr\(^{308}\)-phosphorylated Akt/PKB. The stimulation was time-dependent being maximal at 2 min (+400%), after 15-min exposure of enterocytes to PTH, Akt/PKB phosphorylation reached values near basal. Basal values of Akt/PKB Thr phosphorylation in aged rats were also higher than in young animals, affecting PTH-induced Akt/PKB phosphorylation in aged enterocytes which was greatly diminished (45% above basal levels at 2 min).

Fig. 7. Ageing affects Akt/PKB phosphorylation in PTH-stimulated enterocytes. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with 10\(^{-8}\) M PTH (1–34), for 2 min in the presence or absence of BAPTA-AM (5 µM) or EGTA (0.5 mM). The cells were then lysed and comparable aliquots of lysate proteins were separated by SDS-PAGE followed by Western blotting with anti-Thr\(^{308}\)-phospho Akt/PKB as described under Materials and methods. Total Akt was measured in the same immunoblot by stripping the membrane and reincubating with anti-Akt. Representative images and bar graphs of phospho Akt quantified by scanning densitometry of blots from three independent experiments are shown. *P<0.05, **P<0.025 with respect to the control.

4. Discussion

This study further demonstrates that PTH plays a role in the regulation of duodenal intracellular Ca\(^{2+}\) homeostasis and provides information on the Ca\(^{2+}\) pools that mediate the regulation of intestinal [Ca\(^{2+}\)]\(_i\). In young duodenal cells, the hormone induced a rapid and sustained rise in cytosolic Ca\(^{2+}\) levels [Ca\(^{2+}\)]\(_i\) of rat enterocytes, which involves Ca\(^{2+}\) mobilization from endogenous stores, followed by cation influx from the extracellular milieu finally accounting for the sustained [Ca\(^{2+}\)]\(_i\) phase. A similar profile of changes in [Ca\(^{2+}\)]\(_i\) have been observed in rat enterocytes exposed to 17β-estradiol [42], which stimulates both Ca\(^{2+}\) release from intracellular stores and extracellular Ca\(^{2+}\) influx, and for PTH action in other cell types [43]. In agreement with these observations, it has been previously reported that stimulation of rat enterocytes with PTH results in rapid activation of PI-PLC with a concomitant generation of both IP3 and DAG [13]. The use of specific PLC inhibitors provided additional evidence of the involvement of IP3-sensitive stores in the acute phase of PTH-response in young cells, while Ca\(^{2+}\) entry from out of the cell through VDCC, ligand-gated Ca\(^{2+}\) channels was confirmed with Ca\(^{2+}\) channel blockers. PTH-
induced Ca\(^{2+}\) mobilization from the IP\(_3\)-sensitive stores is blunted in aged duodenal cells. The hormone only stimulates the influx of extracellular Ca\(^{2+}\) through 12 voltage-dependent Ca\(^{2+}\) channels. It is then possible that the PTH-induced activation of PKA [12] and PKC, the latter activated through DAG originated from phospholipids other than PIP2, contribute to the modulation of Ca\(^{2+}\) influx in aged rats. These results are in agreement with previous observations showing that PTH was unable to stimulate PI-PLC activity and thus unable to generate IP\(_3\) and DAG in enterocytes isolated from aged rats [13]. Impairment of PI-PLC activation by the calcitropic hormone 1,25(OH)\(_2\)-vitamin D\(_3\) in enterocytes from aged rats has also been reported [44]. Impairment of PTH-induced Ca\(^{2+}\) mobilization in aged rat duodenal cells may be also the result of a decrease in the number or affinity of the PTH receptors linked to PLC, or alternatively due to inefficient coupling of receptors and guanosine triphosphate (GTP)-binding proteins. In this line of evidence, in duodenal cells of aged animals, the increases required higher PTH concentrations than for young rats. Recently, we have obtained evidence showing that PTH stimulates PI-PLC activity in rat enterocytes via coupling to Gq/11 and the expression of protein Gq/11 decreased with ageing [45]. Additional studies are necessary to ascertain the precise molecular basis underlying the regulation by PTH of cytosolic calcium levels in rat enterocytes searching for understanding if such an uncoupling mechanism is selective to PTH receptor or is a common event occurring in ageing cells. Furthermore, they may contribute to the general understanding of the pathophysiology of ageing as cellular mechanisms that maintain intracellular Ca\(^{2+}\) concentration seem to play a key role during senescence of other cell types [46].

In this study, we analyzed, for the first time, alterations in PI3K activity and response to PTH in rat enterocytes with ageing. PI3K is a heterodimer comprised of one of three catalytic isofoms and one of seven adaptor/regulatory proteins, PI3K, once active, catalyzes the addition of a phosphate moiety specifically to the 3'-OH position of the inositol ring of phosphatidylinositols [47]. The resulting 3'-phosphorylated phosphatidylinositols serve as secondary messengers to activate many downstream signaling targets, initiating the physiological effects of PI3K. Activation of the dimeric p85/p110 PI3K molecule occurs through phosphorylation of a tyrosine residue by either receptor or nonreceptor tyrosine kinases [48]. There are, however, other mechanisms of PI3K activation, as direct interaction between the PI3K catalytic subunit and one or several cellular proteins, such as the complex formation between p21-Ras and p110 resulting in activation of PI3K [49]. The double-enzymatic activity of PI3K (lipid kinase and protein kinase) as well as the ability of this enzyme to activate a number of signal proteins including some oncoproteins determines its fundamental significance in regulation of cell functions such as growth and survival, ageing and malignant transformation [47]. We show here that PI3K is rapidly and transiently activated upon PTH treatment of young rat enterocytes. The hormone activates PI3K inducing the tyrosine phosphorylation of the regulatory (p85\(_a\)) subunit of PI3K. We have obtained evidence that inhibition of the cytosolic tyrosine kinase c-Src prevented PTH-induced phosphorylation of p85\(_a\) [41]. However, how c-Src couples the PTH receptor to PI3K in unknown at present. With ageing, we found that PI3K activity and the tyrosine phosphorylation of its regulatory subunit were severely reduced in rat enterocytes. High basal levels of PI3K activity and p85 phosphorylation in aged rats may be due to activation of anti-apoptotic mechanisms, which control cell survival during ageing, or could be related to mechanical stress during cell isolation. It is unclear whether PI3K activity changes during cell ageing, although a decrease in PI3K activity was reported to cause an increase in ageing rate in normal fibroblasts [50]. However, the mechanism of PI3K-dependent control of cell ageing and the role of individual effectors of PI3K are still unknown. PI3K activity after insulin stimulation was dramatically reduced in liver and muscle of aged rats [51] and insulin signaling defects due to a reduced activation of PI3K and PKB with no changes in the protein levels of the p85 subunit of PI3K has been reported in fat cells from old rats [52]. Moreover, the p85\(_a\) subunit of PI3K in cardiac muscle declined in old mouse whereas the skeletal muscle content of this protein was unaffected by ageing [53].

The catalytic activity of protein and/or lipid kinase regulation is tightly coupled to various extracellular or intracellular signals. Alterations in the levels of intracellular Ca\(^{2+}\) mediate the action of several hormones through the regulation of key enzymes. PI3K activity may be modulated by Ca\(^{2+}\) [54] through a Ca\(^{2+}\)-dependent interaction of calmodulin directly with the p110 catalytic subunit of the enzyme [55], as was also seen with the SH2 domain of the p85 regulatory subunit [56]. Furthermore, Ca\(^{2+}\)-dependent conformational changes of the N-terminal SH2 domain of p85 were reported to be important for PI3K binding to the PDGF receptor [57] and Ca\(^{2+}\) ions were found to directly activate purified PI3-kinases in bovine thymus [58]. Our data clearly indicate that conditions that inhibit PTH-induced increases in [Ca\(^{2+}\)], suppressed, to a great extent, hormone stimulation of PI3K activity and the tyrosine phosphorylation of its regulatory subunit. We have recently reported that PTH-induced increases in cytosolic Ca\(^{2+}\) stimulate the tyrosine phosphorylation of the MAPKs ERK1 and ERK2 [59] and that PI3K is also required for ERK1/2 activation in rat enterocytes [60]. Therefore, it is likely that calcium may play a central role in PI3K-modulation of the MAPK pathway in intestinal cells.

One of the best characterized PI3K effectors is the serine–threonine protein kinase Akt(PKB), whose activation following growth factor or cytokine stimulation is directly dependent on PI3K-derived phosphorylated phosphatidylinositols [61]. The PI3K effector Akt has been implicated, either directly or indirectly, in the phosphor-
ylation and subsequent regulation of several transcription factors [62]. We show here that Akt is also transiently activated by PTH in young rat enterocytes, with similar kinetics of PI3K activation by the hormone. Furthermore, hormone phosphorylation of Akt/PKB was fully dependent on calcium. Phosphorylation of Thr\(^{308}\) by PDK1 in the presence of PIP2 and/or PIP3 is a prerequisite for kinase activation, but phosphorylation of the C-terminal hydrophobic residue Ser\(^{473}\) is required as well for full activation of Akt kinase [63]. With ageing, PTH-stimulation of Akt/PKB is greatly diminished, probably because the system might be near maximal turn on as indicated by the high basal PI3K activity as well as p85 and Akt/PKB phosphorylation. The reasons for the higher basal levels detected in enterocytes from aged rats in comparison to young animals are unclear. Insulin-dependent Akt/PKB phosphorylation was also reduced in muscle tissue and unchanged in liver of aged rats [64] and reduced expression of Akt/PKB was observed in senescent cardiac fibroblasts [65]. Of physiological significance, very recent studies revealed that PI3K is necessary for the functional and morphological differentiation of intestinal epithelial cells [66]. Therefore, as a result of impaired activation of PI3K with ageing, PTH regulation of many biological processes such as cell proliferation and differentiation may be altered.

In summary, our results demonstrate that PTH regulation of enterocyte [Ca\(^{2+}\)]\(_i\) involves Ca\(^{2+}\) mobilization from IP\(_3\)-sensitive stores and the influx of the cation from the extracellular milieu, the former pathway being blunted during ageing. The data also indicates a positive role for intracellular calcium in one of the early signals of PTH in rat enterocytes, the activation of PI3K, and that hormone regulation of PI3K activity and the phosphorylation on Thr\(^{308}\) of its downpressor Akt/PKB is impaired with ageing.

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