Journal of Cellular Biochemistry 86:773–783 (2002)

Involvement of PI3-Kinase and its Association With c-Src in PTH-Stimulated Rat Enterocytes

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Abstract Phosphoinositide-3-kinase (PI3K) is a lipid kinase, which phosphorylates the D3 position of phosphoinositides, and is known to be activated by a host of protein tyrosine kinases. PI3K plays an important role in mitogenesis in several cell systems. However, whether parathyroid hormone (PTH) affects the activity and functional roles of PI3K in intestinal cells remain to be determined. The objective of this study was to identify and characterize the PI3K pathway, and its relation to other non-receptor tyrosine kinases in mediating PTH signal transduction in rat enterocytes. PTH dose- and time-dependently increased PI3K activity with a peak occurring at 2 min. The tyrosine kinase inhibitor genistein, c-Src inhibitor PP1 and two structurally different inhibitors of PI3K, LY294002 and wortmannin, suppressed PI3K activity dependent on PTH. Co-immunoprecipitation analysis showed a constitutive association between c-Src and PI3K, which was enhanced by PTH treatment, suggesting that the cytosolic tyrosine kinase forms an immunocomplex with PI3K probably via the N-SH2 domain of the p85α regulatory subunit. In response to PTH, tyrosine phosphorylation of p85α was enhanced, effect that was abolished by PP1, the inhibitor of c-Src kinase. PTH causes a rapid (0.5–5 min) phosphorylation of Akt/PKB, effect that was abrogated by PI3K inhibitors, indicating that in rat enterocytes, PI3K is an upstream mediator of Akt/PKB activation by PTH. We report here that PI3K is also required for PTH activation of the mitogen-activated protein kinases ERK1 and ERK2. Taken together, the present study demonstrate, for the first time, that PTH rapidly and transiently stimulates PI3K activity and its down effector Akt/PKB in rat enterocytes playing c-Src kinase a central role in PTH-dependent PI3K activation and that PI3K signaling pathway contributes to PTH-mediated MAPK activation. J. Cell. Biochem. 86: 773–783, 2002. © 2002 Wiley-Liss, Inc.

Key words: PTH; PI3K; Akt/PKB; c-Src; MAPK; rat enterocytes; signal transduction

Parathyroid hormone (PTH) is a classical hormone that plays a central role in the regulation of calcium and phosphate metabolism. The expression of PTH receptors, which belong to the family of G-protein coupled receptors [Juppner et al., 1991; Philbrick et al., 1996], has been demonstrated in many tissues [Ito and Ohtsuru, 1996], including the gastrointestinal tract [Selvanayagam et al., 1991; Usdin et al., 1995]. The transduction of PTH signal through the plasma membrane of rat intestinal cells (enterocytes) involves both a Gs-mediated stimulation of adenylyl cyclase (AC) with cAMP production and PKA activation [Picotto et al., 1997] and a Gq-mediated activation of phospholipase Cβ, leading to generation of inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), followed by activation of PKC [Gentili et al., 2000; Massheimer et al., 2000]. PTH also increases intracellular Ca^{2+} levels in rat enterocytes by promoting an initial acute IP_3-mediated mobilization of Ca^{2+} from a thapsigargin-sensitive store and a sustained phase due to Ca^{2+} influx through voltage-dependent Ca^{2+}-channels [Picotto et al., 1997; Massheimer et al., 2000; Gentili et al., unpublished communications]. Furthermore, PTH rapidly and in a dose-dependent fashion stimulates the tyrosine phosphorylation of several enterocyte proteins [Balogh and de Boland, 1999], among which one two the major targets of the hormone could be immunochemically identified as PLCγ and

Grant sponsor: Agencia Nacional de Promocion Cientifica y Tecnologica; Grant sponsor: Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET); Grant sponsor: Ministerio de Salud and Universidad Nacional del Sur, Argentina.

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Received 22 May 2002; Accepted 25 May 2002

DOI 10.1002/jcb.10264

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the growth-related protein mitogen-activated protein (MAP) kinases (p42/44-MAPK), also known as extracellular signal-regulated kinases (ERK1/2). Moreover, initial studies on the mechanisms underlying PTH activation of the enterocyte tyrosine phosphorylation revealed that the hormone stimulates Src tyrosine kinase activity, and consequently, downstream activates PLCγ [Gentili et al., 2000] and the $p21^{ras}$/MAPK pathway [Gentili and de Boland, 2000]. In addition, recent evidence shows that cAMP and Ca^{2+} play a role upstream in the signalling mechanism leading to MAPK activation by PTH in these cells [Gentili et al., 2001]. Of physiological significance, in agreement with the mitogenic role of the MAPK cascade, PTH increased enterocyte DNA synthesis [Gentili et al., 2001].

Phosphatidylinositol-3′-kinase (PI3K) is a member of a subfamily of lipid kinases implicated in many physiological processes, including regulation of cell growth, proliferation, survival and differentiation, vesicle trafficking, glucose transport, platelet function, and cytoskeletal remodeling [Fruman et al., 1998; Wymann and Pirola, 1998]. P85/p110 (class Ia) PI3K is activated by phosphorylation on tyrosine residues in response to many growth factors and cytokines, by receptors with intrinsic tyrosine kinase activity or by receptor-associated tyrosine kinases. A heterodimer comprised of one of three catalytic isoforms 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 PIs [Fruman et al., 1998; Wymann and Pirola, 1998]. The resulting 3′-phosphorylated PIs serve as secondary messengers to activate many downstream signaling targets, initiating the physiological effects of PI3K. There is not information regarding PTH activation of this signaling pathway.

In the present study, we further examined PTH signal transduction in rat enterocytes and evaluated whether the hormone activates PI3K. We also investigated the mechanisms responsible for PI3K activity, and whether this pathway contributes to PTH-induced MAPK activation.

MATERIALS AND METHODS

Chemicals

Synthetic rat PTH (1–34), Immobilon P (Polyvinylidene difluoride, PVDF) membranes and PI were from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-PI3K (anti-p110β and anti-p85α), anti-c-Src, anti-phosphoAkt, anti-phosphotyrosine, and anti-active (phospho)-MAPK were from Promega (Madison, WI). Secondary antibody goat anti-rabbit horse radish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminescence (ECL) were obtained from Amersham Corp. (Arlington Heights, IL), LY294002 and wortmannin were form Alomone Labs (Jerusalem, Israel). All other reagents were of analytical grade.

Animals

Male Wistar rats (3-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, 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 KH_2PO_4, 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 $750 \times g$ for 10 min, washed twice with 154-mM NaCl, 10-mM NaH_2PO_4, 5.6-mM glucose, pH, 7.4, and resuspended in the incubation medium (solution C): 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 prepara-

tions revealed no morphological differences between enterocytes isolated from young and old rats as in previous studies [Massheimer et al., 1999].

In Vitro Treatments

Isolated duodenal cells were pre-equilibrated in solution C for 15 min, and then exposed for short intervals (30 s–10 min) to PTH (10^{-7}–10^{-11} M), agonists or vehicle alone. After treatment, enterocytes were lyzed in 50-mM Tris-HCl, pH 7.4, 150-mM NaCl, 2-mM EGTA, 25-mM NaF, 0.2-mM sodium orthovanadate, 1-mM phenylmethylsulfonyl fluoride, 2-μg /ml leupeptin, 2-μg /ml pepstatin, 2-μg /ml aprotinin, 0.25% sodium deoxycholate, and 1% NP40. Insoluble material was pelleted in a microcentrifuge at 14,000 × g for 10 min. The protein content of the clear lysates was determined according to Lowry [Lowry et al., 1951].

Immunoprecipitation

Enterocytes were treated with PTH and then lyzed. Lysates 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 five 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) and then subjected to Western blot analysis.

Western Blot Analysis

Proteins were separated by one-dimensional SDS–PAGE [Laemmli, 1970]. 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-T buffer (20-mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 containing 5% skim milk) for 2 h to block non-specific binding. Anti-phospho MAPK, anti-cSrc, anti-PI3K (anti-p110β and anti-p85α), anti-phospho Akt/PKB (thr 308), or anti-phosphotyrosine antibodies were allowed to react with the membrane overnight at 4°C. After washing 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, Amersham Corp.), according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

PI3K Assay

PI3K were immunoprecipitated from control or PTH-stimulated cells (700-μg protein) with anti-phosphotyrosine or anti-c-Src antibodies as described above. The immunoprecipitates were washed three times with ice-cold lysis buffer and twice with kinase buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM NA orthovanadate) and then incubated in kinase buffer (50 μl), containing 0.2-mg/ml PI at 37°C for 10 min. The assay was initiated by adding 20-μCi [γ^{32}P]ATP (10-Ci/mmol), and 20-mM $MgCl_2$, 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).

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 ± standard deviation (SD) from the indicated set of experiments.

RESULTS

To investigate the effect of PTH on p110/p85 PI3-kinase, isolated rat enterocytes were briefly stimulated with the hormone (10^{-8} M) and PI3-kinase activity was measured after immunoprecipitation of the cell lysates with anti-phosphotyrosine. Assays with PI as a substrate

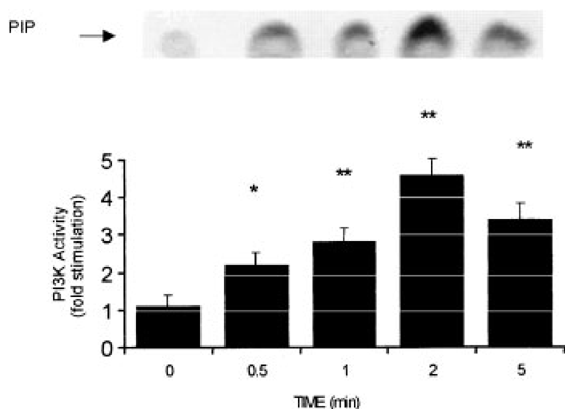

Fig. 1. Kinetics of PI3K activation by PTH. Cell lysates (700 μg of protein) from control and PTH (10^{-8} M)-stimulated cells, were immunoprecipitated with an antibody against P-tyrosine and immunoprecipitates were assayed for PI3K activity as described under Materials and Methods section. A representative image of the phosphorylated substrate (PIP) is shown (top). Means ± SD of fold activation of PI3K are plotted as a function of the duration of PTH stimulation of cells after scanning the autoradiographic films exposed to TLC plates from three independent experiments (bottom). $^{*}P < 0.05$, $^{**}P < 0.025$ respect to the control (0).

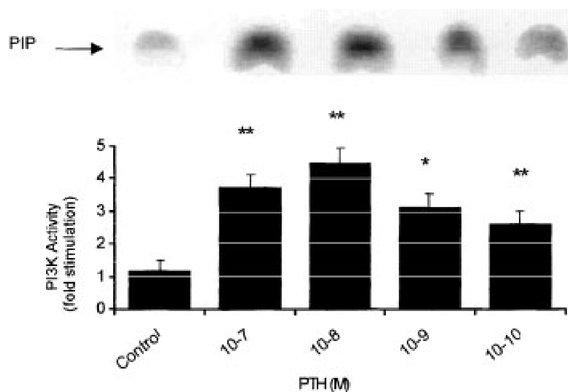

Fig. 2. Dose-dependent stimulation of PI3K activity by PTH. Enterocytes were treated with PTH at the concentrations indicated for 2 min and lyzed. PI3K was immunoprecipitated, and kinase activity was determined as described under Materials and Methods section. A representative image and bar graphs representing intensities of the phosphorylated substrate (PIP) quantified by scanning densitometry of chromatography plates from three independent experiments are shown. $^{*}P < 0.05$, $^{*}P < 0.025$, respect to the control (C).

revealed that the lipid kinase activity of PI3K was rapidly and transiently induced upon PTH treatment of enterocytes, peaking after about 2 min (approximately fourfold) (Fig. 1). The constitutive activity of PI3K observed in untreated cells is most likely due to mechanical stress during cell isolation. The effects of the hormone were dose-dependent (10^{-11}–10^{-7} M) with maximal stimulation achieved at 10^{-8} M (Fig. 2). Pre-incubations of enterocytes with genistein (100 μM), a tyrosine kinase inhibitor, completely abolished PTH-stimulated PI3-kinase activity, while daidzein, an inactive analog of genistein, did not block PI3K activity dependent on PTH (Fig. 3). To go further into the mechanism that relay the signal from PTH receptor to PI3-kinase, and since, the hormone activates the cytosolic tyrosine kinase, c-Src, in rat enterocytes [Gentili et al., 2000], we examined the contribution of c-Src in the PI3-kinase activation induced by PTH. As shown in Figure 4, the inhibitor of c-Src kinases, PP1 (10 μM), suppressed hormone-dependent PI3K activity, confirming a role of c-Src kinases in this signaling cascade. PTH stimulation of PI3-kinase activity was also effectively blocked by two commonly used PI3-kinase inhibitors, LY294002 (20 μM) (Fig. 4) and wortmannin (100 nM) (not shown). To examine whether PI3-kinase activity was detected in association with c-Src after PTH stimulation, PI3-kinase was measured in anti-c-Src immunoprecipitates from enterocytes treated with 10^{-8} M PTH. As shown in Figure 5, an increase in PI3-kinase activity was detectable at 30 s, and reach a peak value within 2 min of treatment (fourfold) after hormone addition, decreasing near basal values at 5 min. Thus, a time-dependent increase in PI3-kinase activity was observed in anti-c-Src precipitates after PTH stimulation, further

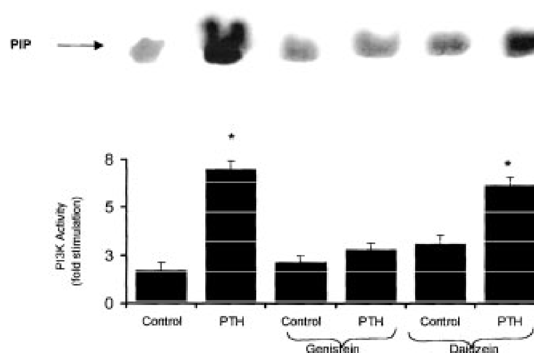

Fig. 3. Role of tyrosine phosphorylation in the stimulation of PI3K activity by PTH. Enterocytes were treated with 10^{-8} M PTH during 2 min in the absence or presence of genistein (100 μM) or daidzein (100 μM), and lyzed. PI3K was immunoprecipitated, and kinase activity was determined as described under Materials and Methods section. A representative image and bar graphs representing intensities of the phosphorylated substrate (PIP) quantified by scanning densitometry of chromatography plates from three independent experiments are shown. $^{*}P < 0.025$, respect to the corresponding control (C).

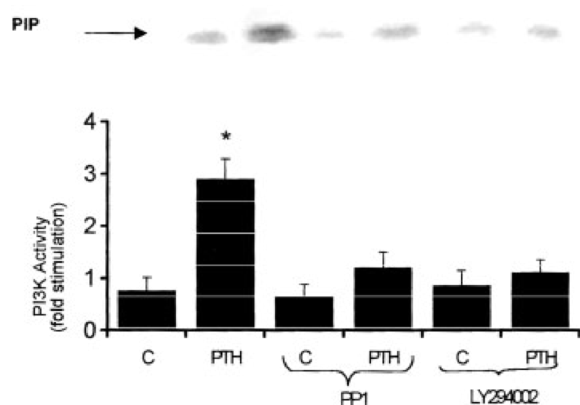

Fig. 4. Suppresion of PTH-stimulated PI3K activity by the c-Src kinase inhibitor PP1 and LY294002. Enterocytes were treated with 10^{-8} M PTH during 2 min in the absence or presence of PP1 (10 μM) or LY294002 (20 μM), and lyzed. PI3K was immunoprecipitated, and kinase activity was determined as described under Materials and Methods section. A representative image and bar graphs representing intensities of the phosphorylated substrate (PIP) quantified by scanning densitometry of chromatography plates from three independent experiments are shown. $*P < 0.025$, respect to the corresponding control (C).

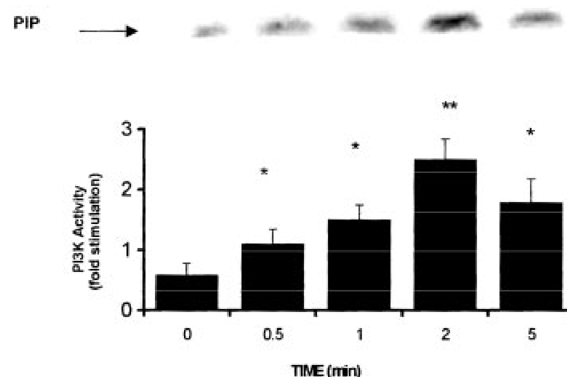

Fig. 5. Activation of PI3K in anti-cSrc immunoprecipitates following PTH stimulation. Enterocytes were treated with 10^{-8} M PTH for the indicated times. Cell lysates were immunoprecipitated with an antibody against c-Src and immunoprecipitates were assayed for PI3K activity as described under Materials and Methods section. A representative image and bar graphs representing intensities of the phosphorylated substrate (PIP) quantified by scanning densitometry of chromatography plates from three independent experiments are shown. $*P < 0.05$, $**P < 0.025$, respect to the control (0).

suggesting that c-Src is a potential mediator of PI3-kinase by PTH. To further analyze whether c-Src associates with PI3-kinase following PTH stimulation, immunoprecipitates from cell lysates obtained with an anti-c-Src antibody were immunoblotted with anti-PI3K antibodies, directed against the 110- and the 85-kDa subunits (Fig. 6A). PI3K was already associated

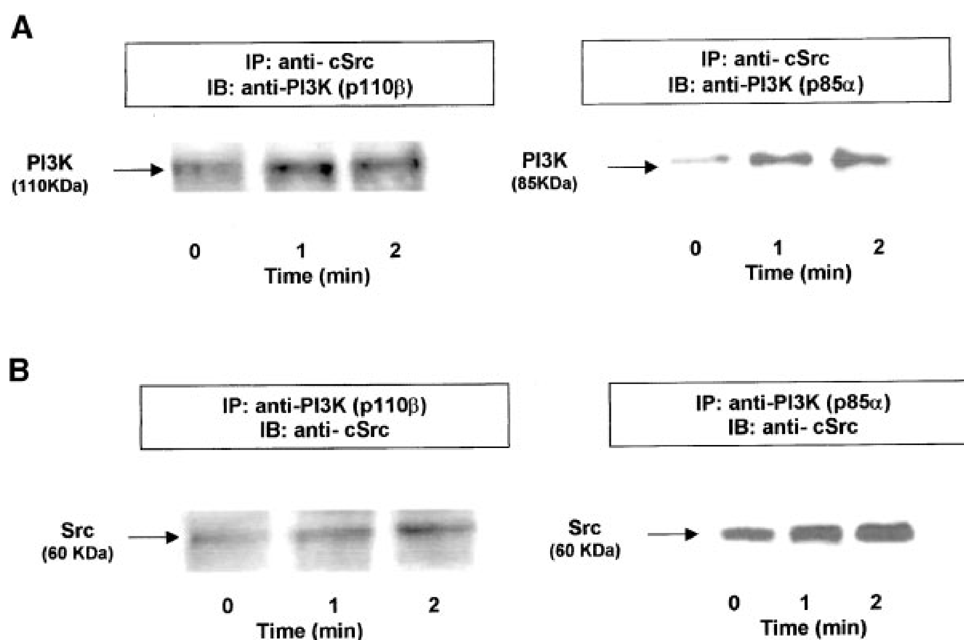

Fig. 6. PTH increases the association of PI3K with c-Src. Enterocytes were treated with 10^{-8} M PTH during 1 and 2 min. **Panel A**: Cell lysates were immunoprecipitated with anti-cSrc antibody, resolved on 10% SDS–PAGE gels, followed by Western blotting with anti-PI3K antibodies (p110β and p85α), as described under Materials and Methods section. **Panel B**: As in Panel A, but immunoprecipitation was performed with anti-PI3K antibodies and immunoblotting with anti-Src antibody as described. ''0'' time-point was obtained in the presence of vehicle; in either panel, no time-dependent changes were observed in vehicle-treated cells. A representative immunoblot of three independent experiments is shown.

with c-Src under basal conditions, the amount of a 110- and 85-kDa proteins recognized by anti-p110β and anti-p85α antibodies were increased by twofold in the anti-c-Src precipitates after PTH stimulation for 1 and 2 min. This association was also confirmed by immunoblot using an anti-c-Src antibody in anti-p110β and anti-p85α immunoprecipitates (Fig. 6B). In order to evaluate whether PTH affects the tyrosine phosphorylation of the p85α regulatory subunit, cells lysates obtained from either PTH-treated or untreated enterocytes were immunoprecipitated with anti-p85α antibody, resolved in SDS–PAGE gels, and then immunoblotted with anti-phosphotyrosine antibody. As shown in Figure 7, consistent with the above data, incubation of enterocytes with PTH increased the level of tyrosine phosphorylation of the p85α regulatory subunit of PI3K. The kinetics for this change in phosphorylation were similar to those found for the increase in kinase activity. PTH-induced tyrosine phosphorylation of p85α was effectively suppressed by the c-Src inhibitor PP1. Similar results were obtained when control assays were carried out adding the antibodies in reverse order: cell lysates were immunoprecipitated with anti-phosphotyrosine and blotted with the anti-p85α antibody (not shown).

An important downstream effector of PI3-kinase is the serine-threonine kinase Akt, or protein kinase B (PKB). The ability of PTH to stimulate the phosphorylation of Akt/PKB was, therefore, investigated. Treatment of enterocytes with PTH led to the rapid (within 30 s) phosphorylation of Akt/PKB on thr 308, 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 (fivefold). Pre-treatment with the PI3-kinase inhibitors, LY294002 (20 μM) or wortmannin (100 nM), completely abrogated PTH-stimulated Akt/PKB thr 308-phosphorylation (Fig. 8).

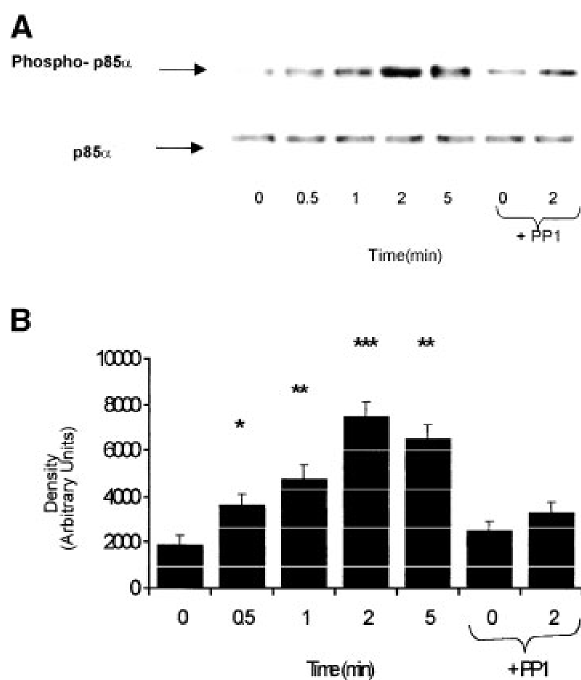

Fig. 7. c-Src phosphorylates the PI3K p85α regulatory subunit in PTH-stimulated enterocytes. Enterocytes were treated with 10^{-8} M PTH, in the presence or absence of PP1 (10 μM), for the indicated times. Cell lysates were immunoprecipitated with anti-p85α, resolved on 10% SDS–PAGE gels, followed by Western blotting with anti-P tyrosine antibody as described under Materials and Methods section. Total p85α was measured in the same immunoblot by stripping the membrane and re-incubating with anti-p85α. (**A**) Representative immunoblot. (**B**) Bar graphs represent intensities of phospho-p85α quantified by scanning densitometry of blots from three independent experiments. *$P < 0.05$, **$P < 0.025$, ***$P < 0.01$ with respect to the corresponding control.

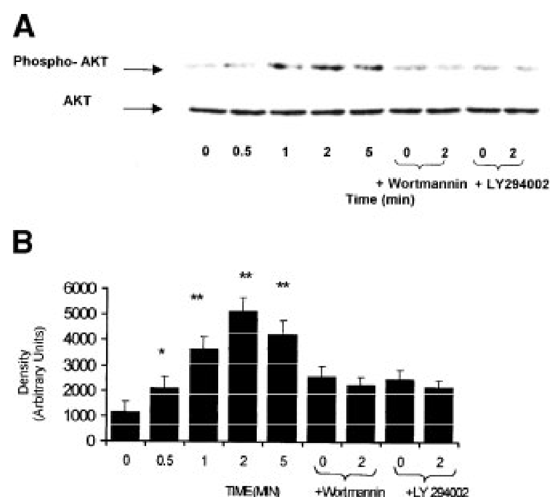

Fig. 8. Akt/PKB phosphorylation in PTH-stimulated enterocytes. Enterocytes were treated with 10^{-8} M PTH, in the presence or absence of Wortmannin (100 nM) or LY294002 (20 μM) for the indicated times. The cells were then lyzed and comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-thr 308-phospho Akt/PKB as described under Materials and Methods section. Total Akt/PKB was measured in the same immunoblot by stripping the membrane and re-incubating with anti-Akt/PKB. (**A**) Representative immunoblot. (**B**) Bar graphs represent intensities of phospho-Akt/PKB quantified by scanning densitometry of blots from three independent experiments. *$P < 0.05$, **$P < 0.025$ with respect to the control (0).

PI3K has been reported to be an upstream regulator of the extracellular regulated-mitogen-activated protein kinases ERK1 and ERK 2 [Cross et al., 1994; vonWillebrand et al., 1996; Jascur et al., 1997; Sharma et al., 1998; Conway et al., 1999]. Our previous demonstration that PTH lead to the phosphorylation and activation of ERK1 and ERK2 [Gentili and de Boland, 2000], prompted us to investigate a possible role for PI3K in the regulation of MAPKs by PTH. To address this issue, cells were stimulated with the hormone (10^{-8} M) in the presence of PI3K inhibitor LY294002, and the activity of ERK1 and ERK2 was assessed by immunoblotting cell lysates with antibodies that detect the dual phosphorylated (activated) form of both ERK1 and ERK2. The results in Figure 9, demonstrate that pre-incubation with LY294002 decrease the ability of PTH to induced ERK1 and ERK2 activation. The membranes were stripped and reprobed with anti-ERK antibodies to assess loading of the gels. These results suggest that the PI3K pathway contribute to PTH stimulation of the MAPK signaling cascade in rat enterocytes.

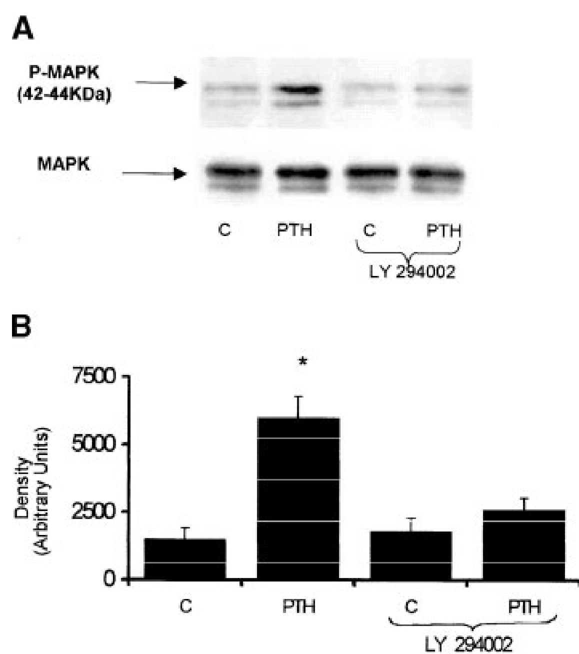

Fig. 9. Inhibition of PI3K activity with LY294002 results in specific inhibition of MAPK phosphorylation in response to PTH. Enterocytes were treated with 10^{-8} M PTH during 1 min in the presence or absence of 20-μM LY294002. The cells were then lyzed and comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-active (phospho-)MAP kinase as described under Materials and Methods section. Total MAPK was measured in the same immunoblot by stripping the membrane and re-incubating with anti-total MAPK (ERK1/2). (**A**) Representative immunoblot. (**B**) Bar graphs represent intensities of both phospho p42 and phospho p44 MAPK quantified by scanning densitometry of blots from three independent experiments. $*P < 0.025$ with respect to the corresponding control (C).

DISCUSSION

The data in the present study demostrates that p85/p110 PI3 kinase is rapidly and transiently activated upon PTH treatment of rat enterocytes, with a pattern of activation typical of other PI3K inducers [Ruderman et al., 1990; Rordorf-Nikolic et al., 1995; Sizemore et al., 1999]. The activation of PI3K is a novel signal in response to the hormone, and presents a new perspective in understanding the full function of the PTH receptors. Class Ia PI3Ks have been found to be associated with and activated by a large number of oncogene products, growth factor receptors, and non-receptor tyrosine kinases of the Src family [Cantley et al., 1991]. The p85 familiy of regulatory subunits of PI3K forms a stable heterodimeric complex with the p110 catalytic subunit. This complex provides thermal stability to p110, but also suppress its activity. Binding of the two SH2 domains of p85 mediates localization to the membrane, where PI3K substrates reside, by directly interacting with phosphotyrosine residues on activated receptors or adaptor proteins [Fantl et al., 1992; Kashishian et al., 1992]. This interaction also relieves the inhibitory effect of the regulatory subunit without dissociating the complex. Binding to GTP-ras also facilitates localization at the membrane and activation [Fruman et al., 1998]. Class Ib (PI3Kγ) differs from class Ia enzymes because of its ability to be directly activated by βγ subunits of heterotrimeric G proteins [Fruman et al., 1998]. Interestingly, a p85/p110 type PI3K activity was also shown to be activated by βγ subunits alone [Zhang et al., 1995] or in combination with phosphopeptides [Tang and Downes, 1997], and also by Ca^{2+}-calmodulin via interaction with p85 SH2 domains [Joyal et al., 1997].

Activation of enterocyte c-Src kinase is an early event in PTH signaling [Gentili et al., 2000]. Our data provide evidence that p85/p110 PI3 kinase, participates in signalling pathways, involving members of the Src family of non-receptor tyrosine kinases. We have detected an increased PI3K activity in anti-c-Src precipitates in response to PTH, with kinetics corre-

lated to those observed for the activation of c-Src signaling [Gentili et al., 2000]. PP1, an inhibitor of c-Src kinase [Hanke et al., 1996] abolished PI3K activity, suggesting that c-Src lie upstream of the PI3K in PTH-treated cells. Our finding that c-Src and PI3K co-immunoprecipitate in a PTH-dependent fashion from enterocytes is in agreement with similar findings in thrombin-stimulated platelets [Gutkind et al., 1990], insulin-like growth factor I-stimulated glial cells [Pomirance et al., 1995], in osteoclast treated with colony-stimulating factor I [Grey et al., 2000], and in cholecystokinin-stimulated pancreatic acinar cells [Nozu et al., 2000]. Furthermore, inhibition of c-Src kinase prevented PTH-induced phosphorylation of the p85α regulatory subunit of PI3K. How c-Src couple the PTH receptor to PI3K is unknown, it is possible that upon activation can directly recruit PI3K [Prasead et al., 1993; Pleiman et al., 1994]. Alternatively, c-Src could phosphorylate docking/scaffolding proteins, which contain binding sites for the SH2 domains of the p85 subunit of PI3K [Lee and States, 2000].

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 PIs [Chan et al., 1999]. The PI3K effector Akt has been implicated, either directly or indirectly, in the phosphorylation and subsequent regulation of several transcription factors [Brunet et al., 1999; Madrid et al., 2000]. We show here, for the first time that Akt is also transiently activated by PTH in rat enterocytes, as measured by Western blot analysis of Thr 308-phosphorylated Akt, with similar kinetics of PI3K activation by the hormone. Phosphorylation of Thr 308 by PDK1 in the presence of PIP2 and/or PIP3 is a pre-requisite for kinase activation, but phosphorylation of the C-terminal hydrophobic residue Ser 473 is required as well for full activation of Akt kinase [Vanhaesebroeck and Alessi, 2000]. The mechanisms of Akt activation by G-protein coupled receptors are complex, some of which appear to be PI3K independent. Moreover, agents that enhance the intracellular levels of cAMP [Sable et al., 1997] and arachidonic acid [Gorin et al., 2001] have been shown to activate Akt by a PI3K-independent mechanism. In rat enterocytes, Akt activation by PTH appears to be fully dependent on PI3K, as hormone-induced Akt phosphorylation was completely suppress by the PI3K inhibitors, wortmannin, which irreversibly inhibits the catalytic subunit of class I PI3Ks [Wymann et al., 1996], and the unrelated compound, LY294002, by a reversible mechanism [Vlahos et al., 1994].

In addition to their lipid kinase activity, certain PI3Ks also exhibit protein kinase activity that have been implicated in the regulation of the MAPK cascade. Recent studies have shown that inhibitors of PI3K lead to inhibition of agonist-stimulated ERK1 and 2 activation [Cross et al., 1994; vonWillebrand et al., 1996; Conway et al., 1999], and different forms of dominant negative p85 mutants also inhibit agonist-stimulated ERK activation [vonWillebrand et al., 1996; Jascur et al., 1997; Sharma et al., 1998]. However, it has also been reported that PI3K inhibition does not influence ERK activity [Ferby et al., 1996; Scheid and Duronio, 1996]. The MAP kinase pathway is initiated after activation of receptor or non-receptor tyrosine kinases (e.g., Src) resulting in tyrosine phosphorylation of Src proteins, and their association with Grb2 and Sos. Sos in turn recruits the GTP-binding protein, Ras, to the signaling complex. Ras then brings Raf, the first kinase of the MAPK cascade, to the membrane, leading to sequential phosphorylation and activation of MEK and MAPK [Seger and Krebs, 1995]. Stimulation of MAP kinase may occur through activation of G protein-coupled receptors by stimulation of Src kinases or by direct signaling to Raf via PKC [Selbie and Hill, 1998]. It is not entirely clear, how the MAPK cascade is regulated by PTH in rat enterocytes, although c-Src [Gentili and de Boland, 2000], calcium, and the AC/cAMP/PKA pathway [Gentili et al., 2001] have been found to contribute to MAPK activation. The connection of PI3K to the MAPK pathway and its role in PTH transduction has not been explored. The fact that LY294002 diminished PTH-induced ERK1 and ERK2 phosphorylation, demonstrates that PI3K is also required to allow MAPK activation after hormone stimulation in rat enterocytes. The precise mechanism by which PI3K regulates MAPK activity in rat enterocytes is unknown. In some cell types, PI3K functions upstream or downstream effector of Ras, an this is still an area of some controversy [Hu et al., 1995; Kauffmann-Zeh et al., 1997; Wennstrom and Downward, 1999].

PI3K have also been implicated in controlling DNA synthesis and the cell cycle [McIlroy et al., 1997]. Microinjection studies demonstrated a requirement of p110α activity in platelet-derived growth factor-stimulated proliferation of 3T3 cells [Roche et al., 1994], and activation of an inducible version of p110α is sufficient to induce progression of cells through G1 and into S phase [Klippel et al., 1998], and in IL-2 signaling, inhibition of PI3K results in lack of activation of the transcription factor E2F [Brennan et al., 1997]. Of physiological significance, in agreement with the well-known participation of MAPK in pathways leading to mitogenic effects, we previously demonstrated that PTH activation of the MAPK cascade in rat enterocytes culminates in translocation of activated ERK1 and ERK2 to the nucleus, which leads to a stimulation of duodenal cell proliferation [Gentili et al., 2001]. Thus, it is possible that the growth-promoting effects of PTH on enterocytes could be mediated, at least in part, via the activation of PI3K. Additional experiments will be required to define the point at which signals emanating from PI3K intersect with the MAPK cascade and the proliferative response of PTH in intestinal cells.

In summary, our results demonstrate for the first time that PTH, a G protein-coupled receptor agonist, rapidly and transiently stimulates p85/p110 PI3K activity, and its down-effector Akt/PKB in rat enterocytes, and that c-Src kinase played a central role in PTH-dependent PI3K activation. In addition, we showed that PI3K signaling pathway contributes to PTH-mediated MAPK activation in these cells.

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