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Gastrointestinal growth factors and hormones have divergent effects on Akt activation

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ABSTRACT

Akt is a central regulator of apoptosis, cell growth and survival. Growth factors and some G-protein-coupled receptors (GPCR) regulate Akt. Whereas growth-factor activation of Akt has been extensively studied, the regulation of Akt by GPCR's, especially gastrointestinal hormones/neurotransmitters, remains unclear. To address this area, in this study the effects of GI growth factors and hormones/neurotransmitters were investigated in rat pancreatic acinar cells which are high responsive to these agents. Pancreatic acini expressed Akt and 5 of 7 known pancreatic growth-factors stimulate Akt phosphorylation (T308, S473) and translocation. These effects are mediated by p85 phosphorylation and activation of PI3K. GI hormones increasing intracellular cAMP had similar effects. However, GI-hormones/neurotransmitters [CCK, bombesin, carbachol] activating phospholipase C (PLC) inhibited basal and growth-factor-stimulated Akt activation. Detailed studies with CCK, which has both physiological and pathophysiological effects on pancreatic acinar cells at different concentrations, demonstrated CCK has a biphasic effect: at low concentrations (pM) stimulating Akt by a Src-dependent mechanism and at higher concentrations (nM) inhibited basal and stimulated Akt translocation, phosphorylation and activation, by de-phosphorylating p85 resulting in decreasing PI3K activity. This effect required activation of both limbs of the PLC-pathway and a protein tyrosine phosphatase, but was not mediated by p44/42 MAPK, Src or activation of a serine phosphatase. Akt inhibition by CCK was also found *in vivo* and in Panc-1 cancer cells where it inhibited serum-mediated rescue from apoptosis. These results demonstrate that GI growth factors as well as gastrointestinal hormones/neurotransmitters with different cellular basis of action can all regulate Akt phosphorylation in pancreatic acinar cells. This regulation is complex with phospholipase C agents such as CCK, because both stimulatory and inhibitory effects can be seen, which are mediated by different mechanisms.

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1. Introduction

The members of the Akt family of protein kinases, including Akt1, Akt2 and Akt 3, have been shown to regulate multiple biological functions including apoptosis, cell survival, protein synthesis and glycogenesis [1–4]. Structurally, Akt contains a N-terminal pleckstrin homology domain (which has been shown to bind phosphoinositides), a catalytic domain related to protein kinases A and C (hence the alternative name of PKB) and a C-terminal regulatory region [2]. Two phosphorylation sites are involved in Akt activation: T308 in the catalytic domain and S473 at the C-terminus. After stimulation by insulin or growth factors, both sites are phosphorylated involving a PI3K-dependant mechanism: T308 is phosphorylated by PDK1 (3-phosphoinositide-dependent protein kinase 1) and S473 by a yet unidentified kinase [5]. Akt activation is highly correlated with the S473 phosphorylation level [5–7]. After stimulation by growth factors, generation of 3-phosphoinositides at the plasma membrane cause pleckstrin-domain-mediated translocation of PDK1 and Akt to the membrane, resulting in T308 and S473 phosphorylation and Akt activation [8].

Growth factors almost uniformly activate Akt and the underlying mechanisms have been well studied [9–11]. In contrast, with most G-

Abbreviations: GI, gastrointestinal; GPCR, G protein-coupled receptor; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PDK1, 3-phosphoinositide-dependent protein kinase 1; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; LHRH, luteinizing hormone releasing hormone; CCK, cholecystokinin; JMV, CCK-JMV-180; PIP_3, phosphatidylinositol-3-phosphate; PLC, phospholipase C; DAG, diacylglycerol; cAMP, cyclic AMP; TPA, 12-O-tetradecanoylphorbol-13-acetate; HGF, hepatocyte growth factor; EGF, epidermal growth factor.

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protein coupled receptors and especially with those mediating the actions of gastrointestinal hormones/neurotransmitters, their effects on Akt activation have been much less well studied and the mechanisms of their effects on Akt activation are largely unknown. Activation of some G-protein coupled receptors has been reported to activate Akt in some studies [1,7,12–17], while others report inhibition of Akt by these stimulants [1,18–20]. In some cases such as with the GI hormone/neurotransmitters, cholecystokinin/gastrin, they are reported to stimulate Akt activation in some tissues, inhibit Akt activation in others and not to alter Akt activity in still others [7,17,21–24]. The cellular mechanisms involved in these differences are unclear.

Pancreatic acinar cells are an excellent model for studying the effect of growth factors and G-protein coupled receptor agonists for gastrointestinal hormones/neurotransmitters, because these cells have been shown to express a number of different G protein-coupled receptors for gastrointestinal hormones/neurotransmitters and growth factors functionally connected to multiple signal transduction pathways [25–32]. Dispersed pancreatic acini can be prepared from intact pancreas that are responsive to a number of stimulants, displaying activation/inhibition of multiple signal transduction pathways and changes in cellular function, which allow their cellular basis of action to be studied *in vitro* [25–27,29–31]. Therefore, we choose to study the effect of growth factors and gastrointestinal hormones on Akt activation in pancreatic acini. The examination of their actions in pancreatic acinar cells is also important because PI3K/Akt signaling has been shown to play a central role in pancreatic regeneration [33] and pathological responses of pancreatic acinar cells to CCK [34], but the underlying mechanisms remain unclear. Furthermore, both aberrant signaling through growth factors or gastrointestinal hormones and Akt have been reported to occur in pancreatic pathologies, including cancer and pancreatitis [35–40].

Therefore, the aim of the present study was two-fold. First, to examine the ability of various important gastrointestinal growth factors and gastrointestinal hormones/neurotransmitters to alter Akt activation in a well-established experimental model using primary, non-transfected and non-transformed gastrointestinal cells (i.e. pancreatic acini) and to elucidate the signaling cascades causing the effect on Akt activity by these pancreatic acinar cell stimulants. Furthermore, the signaling cascade for the effects of the GI hormone/neurotransmitter, CCK on Akt activation was studied in detail because of its importance as physiological regulator of pancreatic function as well as its importance in causing experimental pancreatitis, which is a widely used model to study this clinical disorder [21,41].

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–250 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (NIH), Bethesda, MD. Rabbit anti-phospho-Akt-pT308, mouse anti-phospho-Akt-pS473, rabbit anti-Akt antibodies, rabbit anti-phospho-Src family (Y416), mouse anti-phospho p44/42 MAPK (T202/Y204) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Bovine anti-goat horseradish peroxidase (HRP)-conjugate and anti-rabbit-HRP-conjugate antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-tyrosine antibody (PY20) was purchased from BD Biosciences (San Jose, CA). Rabbit anti-PI3-kinase p85 was purchased from Upstate Biotechnology (Lake Placid, NY). Tris/HCl pH 8.0 and 7.5 were from Mediatech, Inc. (Herndon, VA). 2-mercaptoethanol, protein assay solution and SDS were from Bio-Rad Laboratories (Hercules, CA). $CaCl_2$ and $MgCl_2$ were from Quality Biological, Inc. (Gaithersburg, MD). Dulbecco's phosphate buffered saline (DPBS), glutamine (200 mM), Tris/glycine/SDS buffer (10×), and Tris/glycine buffer (10×) were from Biosource. Minimal essential media (MEM) vitamin solution, basal medium Eagle (BME) amino acids 100x and Tris-Glycine gels were from Invitrogen (Carlsbad, CA). COOH-terminal octapeptide of cholecystokinin (CCK-8), hepatocyte growth factor (HGF), bombesin, insulin-like growth factor 1 (IGF-I), basic fibroblast growth factor (bFGF), vasoactive intestinal peptide (VIP), endothelin and secretin were from Bachem Bioscience Inc. (King of Prussia, PA). EGF, thapsigargin, PDGF, GF109203X, A23187, LY294002, wortmannin, PD98059, U0126, PP2, PP3 and deoxycholic acid were from Calbiochem (La Jolla, CA). Carbachol, insulin, dimethyl sulfoxide (DMSO), 12-O-tetradecanoylphobol-13-acetate (TPA), L-glutamic acid, fumaric acid, pyruvic acid, trypsin inhibitor, acetic acid, HEPES, Triton X-100, TWEEN® 20, phenylmethanesulfonylfluoride (PMSF), EGTA, NaF, $Na_4P_2O_7$, phosphatidylinositol, phosphatidylserine, sucrose, sodium-orthovanadate and sodium azide were from Sigma-Aldrich, Inc. (St. Louis, MO). Albumin standard, Protein G, Super Signal West (Pico, Dura) chemiluminescent substrate and stripping buffer were from Pierce (Rockford, IL). Protease inhibitor tablets were from Roche (Basel, Switzerland). Purified collagenase (type CLSPA) was from Worthington Biochemicals (Freehold, NJ). Nitrocellulose membranes were from Schleicher and Schuell BioScience, Inc. (Keene, NH). [γ^{32}P]ATP (3000 Ci/mmol) was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). TLC plastic Sheets was purchased from EMD Chemicals (Gibbestown, NJ). 2-propanol and chloroform were purchased from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ). Alexa 594-conjugated anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Poly-L-lysine coated slides and sample chambers were from Wescor (Logan, UT).

2.2. Methods

2.2.1. Tissue preparation

Pancreatic acini were obtained by collagenase digestion as previously described [26]. Standard incubation solution contained 25.5 mM HEPES (pH 7.45), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium glutamate, 5 mM sodium fumarate, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM glutamine, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (v/v) amino acid mixture.

2.2.2. Acini stimulation

After collagenase digestion, dispersed acini were pre-incubated in standard incubation solution for 2 h at 37 °C with or without inhibitors as described previously [26,30]. After pre-incubation 1 ml aliquots of dispersed acini were incubated at 37 °C with or without stimulants. Cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml). After sonication, lysates were centrifuged at 10,000 ×g for 15 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent. Equal amounts of samples were analyzed by SDS-PAGE and Western blotting.

2.2.3. Western blotting/immunoprecipitation

Western blotting and immunoprecipitation were performed as described previously [42]. Whole cell lysates, immunoprecipitates or lysates of subcellular fractions were subjected to SDS-PAGE using 10% and 4–20% Tris-Glycine gels. After electrophoresis, protein was transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% Tween® 20, 5% nonfat dry milk) at 4 °C overnight or at room temperature for 1 h. Membranes were then incubated with primary antibody under constant agitation at antibody dilutions suggested by the antibody supplier. After primary antibody incubation membranes were washed twice in blocking buffer for 4 min and then incubated with HRP-conjugated secondary antibody (anti-mouse, anti-rabbit, anti-goat) according to the species of the first antibody for 45 min at

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