Published in final edited form as:

J Pharm Sci Pharmacol. 2014 June 1; 1(2): 160–164.

Modulation of Epidermal Growth Factor Stimulated ERK Phosphorylation and Cell Motility by Inositol Trisphosphate Kinase

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Abstract

Epidermal growth factor [EGF] mediated stimulation of its receptor in endothelial cell [EC] is accompanied by phosphorylation of the EGF-receptor [EGFR] and activation of phospholipase C- γ , resulting in the breakdown of phosphatidylinositol(4,5)-bisphosphate and generating inositol (1,4,5)-trisphosphate [IP₃] and diacylglycerol. IP₃ thus formed can be further converted to inositol (1,3,4,5)-tetrakisphosphate [IP₄] by an enzyme called IP₃-kinase [IP₃K]. In this study we have investigated the effect of modulation of intracellular IP₃K activity by the use of an inhibitor, 2-trifluoromethyl [6-(4-nitrobenzyl)-purine] [IP₃KI] and siRNA against IP₃KB on EGF-induced ERK-phosphorylation and cell motility. EGF stimulated ERK-phosphorylation that has been implicated in EGF-stimulated cell migration was inhibited by both IP₃KI and siRNA against IP₃KB. Inhibition of ERK-phosphorylation was accompanied by decreased cell migration in the presence of IP₃KI.

Keywords

Inositol Phosphates; EGF Receptor; Cell Migration

INTRODUCTION

Epidermal growth factor receptor activated PLC- γ mediated pathway increases cell motility by inducing the cytoskeletal reorganization that is required for asymmetric cell locomotion (Chen et al., 1996). Phospholipase C- γ activation cleaves phosphoinositide 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (Berridge, 1993). Inhibition of PIP₂ breakdown results in failure of lamellipodium extension and cell motility (Chen et al., 1994). The importance of PIP₂ in cell motility was recently demonstrated by finding that detachment of rear of the cell necessary for cell motility requires *m*-calpain activation, which is localized at the rear of the cell in a PIP₂ enriched domain (Shao et al., 2006). EGF-stimulated *m*-calpain activation is mediated through ERK1/2 phosphorylation (Galding et al., 2000) and inhibition of ERK1/2 phosphorylation is accompanied by decreased motility (Galding et al., 2001).

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The open question remains of the function of PLC γ . Is it merely to remove PIP₂ from the membrane mobilizing actin binding proteins and eliminating docking sites, or does it involve downstream signaling molecules? While the role of IP₃ in calcium mobilization is well established (Berridge, 1993), role of other inositol phosphates, besides IP₃, in various aspects of cell signaling are only now being recognized (Miller et al., 2008). Calcium contributes to transcellular contractility (Helfman et al., 1999) needed for motility (Lauffenburger et al., 1996). As IP₃ generated in the cell is further converted to I(1, 3, 4, 5)P₄ by IP₃-kinase (Irvine et al., 2006), we have investigated its function in this publication.

Currently, three isoforms of IP₃-kinase, types—A, B and C are shown to be present in various cell types (Dewaste et al., 2002). In IP₃K-B deficient mice, decreased B-cell survival is associated with decreased phosphorylation of ERK1/2 (Marechal et al., 2007) and in a more recent study, overexpression of IP₃K type A has been associated with altered cytoskeletal reorganization and increased cell motility (Windhorst et al., 2008). In this study, we have investigated the effect of modulation of IP₃K activity with an IP₃-kinase specific inhibitor [IP₃KI] and a siRNA to IP₃K-B on EGF-stimulated ERK-phosphorylation and cell motility.

MATERIALS AND METHODS

Cell Culture

Human microvascular endothelial cells (HMvEC) were grown in MCDB131, media supplemented with 10% FBS, 1 μ g/mL of hydrocortisone and 10 ng/mL of EGF.

Study of ERK1/2 and EGFR Phosphorylations

Cells were quiesced by overnight incubation with 0.1% dialyzed serum for endothelial cells (without EGF). Quiescent cells were washed once and incubated with or without IP₃KI for 4 hrs and then stimulated with EGF (10 nM) for 0 to 20 minutes, as specified in the legend. Incubation terminated with hot lysis buffer. Proteins were separated by gel electrophoresis, transferred to PVDF membrane and probed with primary polyclonal antibodies against actin (Sigma-Aldrich), phosphorylated ERK1/2 (Cell Signaling Technology), and EGFR phosphorylated on Y1173 (Santa Cruz Biotechnology Inc.) and Y1068 (Cell Signaling Technology). The GAPDH (Monoclonal antibody obtained from rabbit) and secondary antibodies were purchased from Sigma-Aldrich.

SiRNA Transfection and IP₃ K Downregulation

Endothelial cells were seeded at 80% confluency. The day after, the medium was removed and replaced by OptiMEM. The cells were then transfected with control siRNA or with 50 pmol of siRNA designed to knock-down IP₃KB (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). After 6 hours, the OptiMEM medium containing the siRNA and the Lipofectamine was removed and the cells were incubated overnight in regular medium (MCDB131). The down regulation of the IP₃KB expression was confirmed by immunoblot (antibody against IP₃KB obtained from Santa Cruz Biotechnology Inc.). The effects of this downregulation on the phosphorylation of ERK1/2 and EGFR were tested as described previously.

Wound Healing Assays

Cell migration studies were done as described by us previously (Chen et al., 1996). Briefly, endothelial cells were grown up to confluency. After an overnight incubation in the quiescent medium, an acellular area (wound) was created by scraping the cells with a pipette tip. The cells were then washed and treated overnight with EGF (10 nM) and increasing concentrations of IP₃KI (Calbiochem, 0 to 20 μ M). Pictures of the wound were taken before and after the overnight incubation. The closure of the wound was estimated using Metamorph software.

RESULTS

We queried whether IP₄ may contribute to EGF-induced motility by determining the effect of IP₃K inhibitor (IP₃KI) on cell migration by *in vitro* wound healing assay (Fig. 1). IP₃KI alone, at 10 μ M and 20 μ M, has no effect on basal cell motility, but it is clearly able to inhibit EGF-stimulated cell migration at the above concentrations.

As EGF-induced motility was blocked, we checked for EGFR signaling. Short-term activation of EGFR stimulation by EGF is accompanied by ERK phosphorylation. This EGF-stimulated ERK-phosphorylation is inhibited by pre-incubation with 20 μ M of IP₃KI (Fig. 2(A)). Dose-dependent nature of this inhibition, with an IC₅₀ between 10–15 micromolar, is further demonstrated in Figure 2(B). This value is comparable to that reported by Chang et al., 2002 in their *in-vitro* experiments on the ability of this compound to inhibit IP₃K.

As this IP₃KI inhibitor was obtained by screening an ATP inhibitor library, there was a possibility that both the above effects of this inhibitor (inhibition of EGF-stimulated ERK-phosphorylation and motility) were due to the inhibition of the EGF-receptor kinase activity rather than IP₃-kinase activity. This possibility was further investigated by studying the effect of IP₃KI on EGFR phosphorylation (Fig. 3).

IP₃KI, not only inhibited EGF-stimulated ERK-phosphorylation as depicted in Figure 1, but it also clearly inhibited EGFR-phosphorylation at the same concentrations (Fig. 3(A)). Inhibitor decreased EGF-stimulated phosphorylation at both Y-1173 and Y-1068 (Fig. 3(B)). Though the extent of phosphorylation at these sites varied from experiment to experiment. This clearly raised the possibility that some or all of the effects of IP₃KI could be explained by its direct inhibition of EGFR-kinase, rather than its inhibition of IP₃K.

As a second, independent approach to eliminating IP₄, and demonstrate that IP₃K is a key signaling molecule contributing to EGF-induced motility, we downregulated the main IP₃K isoform in endothelial cells, IP₃KB, with IP₃KB-specific siRNA (Fig. 4(A)). siRNA to IP₃KB decreased the expression of IP₃KB (Figs. 4(A) comparing lane 1 with 2 and 3 and lane 4 with 5 and 6). Comparing the EGF-stimulated *p*-ERK bands without and with 50 nM siRNA concentrations clearly shows inhibition of phosphorylation with decreased IP₃KB expression (comparing lanes 1, 2, 3 with 4, 5, 6).

DISCUSSION

IP₃KI used in this study, 2-trifluoromethyl (6-(4-nitrobenzyl)-purine, was first reported by Chang et al., (2002). It was derived from screening the library of purine compounds and was suggested to compete for ATP binding site in IP₃K and thereby prevent phosphorylation of IP₃ to IP₄. Chang et al., (2002) reported that addition of this IP₃KI to HeLa cells increased cytosolic calcium concentration over 1–2 min in a dose dependent manner. That was consistent with the inhibition of conversion of IP₃ to IP₄, which was accompanied by increased IP₃-mediated cytosolic calcium increase. It is also possible that IP₄ or downstream metabolite could play a role in inhibition of calcium release [Bird et al., 1996]. In that case IP₃KI by preventing the formation of IP₄ will remove that inhibition and enhance the release of calcium.

Rate limiting step for growth factor-induced chemokinetic movement is rear detachment, brought about by the activation of *m*-calpain [Galding et al., 2000, Galding et al., 2001]. *m*-Calpain activation is achieved via ERK/mitogen-activated protein kinase, via the divergent Ras-Raf-MEK pathway to mediate focal adhesion disassembly. Inhibition of either the PLC- γ or ERK pathway leads to a loss of EGFR-mediated motility [Chen et al., 1996; Shao et al., 2006]. As IP₃KI was shown to inhibit EGF-stimulated ERK phosphorylation in a dose dependent manner, we investigated the effect of this inhibitor on EGF-stimulated cell migration. As shown in Figure 2, IP₃KI alone at 10 and 20 μ M concentration has no effect on endothelial cell migration while it clearly inhibits EGF-stimulated cell migration at both 10 and 20 μ M concentrations.

Interestingly, Windhorst et al., (2008) have reported that IP₃-kinase type A overexpression in H1299 cells resulted in cytoskeletal reorganization as well as increased cell motility. Cell motility but not cytoskeletal reorganization was attributed to the enzymatic activity of IP₃-kinase [Windhorst et al., 2008]. More recently increased expression of IP₃KA increases invasive migration *in vitro* and metastasis in a xenograft SCID mouse model [Windhorst et al., 2010]. If enhanced activity of IP₃K, as a result of IP₃K overexpression results in increased cell motility then our results that inhibition of IP₃K with IP₃KI and downregulation of IP₃KB in our siRNA studies—leads to decreased ERK-phosphorylation accompanied by decreased cell motility would suggest the involvement of higher inositol phosphates in cell migration.

Eva et al., (2012) have recently reported that IP₃-kinase type A activity opposes NGF stimulated neurite outgrowth that is accompanied by attenuation of ERK phosphorylation. Considering all the above studies on different iso-forms of IP₃K's, it appears that while they generally have an effect on cell migration, the exact effect may be tissue and isoform dependent.

Unlike IP₃, whose role as an intracellular mediator of calcium release is well established, significance of IP₄ is far from clear. Original belief that IP₃ kinase's only function to inactivate IP₃, and thereby terminate calcium signaling is certainly incorrect. Several intriguing possibilities have been suggested for the functional significance of IP₄ generated by the action of IP₃K on I(1,4,5)P₃. IP₄ is capable of binding to proteins containing class I

pleckstrin homology domain [Cozier et al., 2000]. In some instances, such as Ras GAP activity of GAP1^{IP4BP} was inhibited by phospholipids, but activated by IP₄ [Cullen et al., 1995]. In proteins with more than one pleckstrin homology domain, binding of IP₄ to one of those sites, modulates the binding of the other PH site to its target [Stricker et al., 2006] including PIP₃. New evidence [Miller et al., 2007] has implicated IP₄ in negative modulation of store operated calcium channel.

When IP₄ is considered as the “soluble version” of PIP₃ and because of its capability to bind to PH domain, depending on the cell type and the specific protein being regulated (i.e., whether it has one or more PH domains), several possible interaction scenarios are possible. In our system, *m*-calpain bound to PIP₂ is localized in the rear of the cell and plays an important role in cell detachment and locomotion [Shao et al., 2006]. Epidermal Growth factor mediated activation of *m*-calpain is mediated through ERK-phosphorylation. In this context it is interesting to note that Burton et al., have demonstrated that IP₃K localization to the cytoskeletal and its further regulation of activity by calmodulin [Lloyd-Burton et al., 2007].

While data reported in this publication are consistent with other recent observations that IP₃K isoenzymes through ERK phosphorylation may modulate cell migration, additional experiments with overexpressed kinase dead mutants of various isoenzymes as well as simultaneous determination of IP₄ levels will be required to prove a causal relationship.

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Figure 1.

Effect of IP₃KI on EGF-stimulated cell migration in endothelial cell—Cells were treated with or without EGF, in presence and absence of IP₃KI inhibitor overnight at 37°C. Migration was measured by the ability of cells to move into the acellular area. *: Significantly different from the control ($p < 0.05$); **: Significantly different from EGF-induced ($p < 0.05$).

Figure 2.

Effect of IP₃KI on EGF stimulated ERK phosphorylation: Quiescent endothelial cells incubated with IP₃KI or DMSO (control) for 4 hrs, followed by 15 min stimulation with EGF. Solubilized protein separated on 10% SDS gel and WB probed with rabbit polyclonal against phosphorylated ERK1/2. (A) Endothelial cells stimulated with 10 nM EGF with or without 20 μ M IP₃KI pre-incubation; (B) pre-incubated with increasing concentrations of IP₃KI for 4 hrs. Shown are representative of three independent experiments.

Figure 3.

Experiments were performed as described in Methods. Cells were pre-incubated for 4 hours in presence or absence of IP₃KI followed by stimulation with EGF [10 nM] for (A) 10 minutes; and (B) varying period of time and western blot probed for *p*-EGFR (Y 1173), *p*-EGFR (Y 1068); *p*-ERK, ERK and actin. Shown are representative of at least three independent replicate gels.

Figure 4.

(A) siRNA transfected into endothelial cells by two different transfection agents. Downregulation of IP₃KB was measured by Western blot. As Lipofectamine produced more consistent down regulation of IP₃KB, it was used for further investigation. (B) Cells were treated with either 0 or 50 pmol of siRNA for IP₃KB downregulation as described in the Methods and treated with or without EGF (10 nM) for 2 or 5 minutes.