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Insulin and ATP stimulate actin polymerization in U937 cells by a wortmannin-sensitive mechanism

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Abstract **ATP and insulin stimulate increases in phosphatidylinositol (3,4,5)-trisphosphate levels in myeloid-derived U937 cells. Quantification of FITC-phalloidin binding by fluorescence-activated cell sorting reveals that both ATP and insulin stimulate actin polymerization with distinctive kinetics in U937 cells. The response to ATP is rapid and dose-dependent with an EC_{50} of 200 nM, and is abolished by pre-incubation with the Ca^{2+} chelator BAPTA-AM. At 800 nM concentration, wortmannin, a potent inhibitor of phosphoinositide 3-kinase (PI3K), blocks the late, but not the early phase of actin polymerization stimulated by 100 nM ATP. Responses elicited by 10 μg/ml insulin are slower, smaller and more transient than responses to ATP, and are inhibited by preincubation with 100 nM wortmannin. Actin polymerization can also be stimulated by thapsigargin, but not by phorbol ester, providing further evidence for a role for Ca^{2+} in actin polymerization. These data implicate distinct Ca^{2+} and PI3K-mediated pathways in the regulation of actin polymerization.**

Key words: Actin polymerization; Phosphoinositide 3-kinase; Phosphatidylinositol (3,4,5)-trisphosphate; Insulin; ATP; Calcium mobilization

1. Introduction

Considerable interest has centered around the role of membrane phosphatidylinositol polyphosphates in mitogenic signaling [1], and more recently in the organization and rearrangement of the actin cytoskeleton that parallel the response to growth factors [2]. A number of recent studies have shown that phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), through both the mobilization of intracellular Ca^{2+} and the direct interaction of PtdIns(4,5)P_2 and $InsP_4$ with actin binding proteins, regulates the nucleation and polymerization of actin (for review see [3]). The role of PI3K, an enzyme that catalyses the formation of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3), in promoting the processes of neurite outgrowth, membrane ruffling, growth factor-dependent mitogenesis and respiratory burst activity in neutrophils has been established [4]. Moreover, recruitment and activation of PI3K has been shown to lie upstream of a particular rearrangement of actin filaments that occurs in response to growth factors, known as membrane ruffling [5,6], which may be mediated through activation of the small GTP-binding protein rac [6,7]. However, a potential role for PI3K in the regulation of the processes of actin nucleation and polymerization remains to be established. Parallel increases in cellular F-actin content with increases in both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 levels have been reported in neutrophils activated by β_2-integrin engagement [8] or fMLP [8], as well as in thrombin receptor-activating peptide (TRAP)-stimulated platelets [9] and phorbol ester-stimulated basophilic leukemia cells [10]. However, in contradiction of this evidence, wortmannin, a potent inhibitor of PI3K, does not inhibit fMLP-stimulated F-actin polymerization in neutrophils [11,12] or TRAP-stimulated actin assembly in platelets [9]. In this study we have demonstrated that stimulation of U937 cells with ATP or insulin results in time-dependent increases in cellular F-actin content, and that actin polymerization in response to these two mitogens is inhibited by wortmannin. Our data suggest that distinct PI3K signalling pathways regulate F-actin polymerization in U937 cells and that responses to ATP are additionally mediated by changes in intracellular free Ca^{2+}, but not diacylglycerol.

2. Materials and methods

2.1. Materials

2.1.1. Reagents. ATP, FITC-conjugated phalloidin, thapsigargin, phorbol myristate acetate (TPA), paraformaldehyde, L-glycine, fatty acid-free bovine serum albumin (BSA) and L-glutamine were purchased from the Sigma Chemical Co. RPMI 1640, fetal bovine serum (FBS) and fetal calf serum (FCS) were purchased from Gibco BRL. BAPTA-AM (1,2-bis(*O*-aminophenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester) was obtained from Molecular Probes Inc. All other chemicals were of reagent grade.

2.1.2. Solutions. U937 balanced salts solution contained (final concentration mM) 140 NaCl, 5 KCl, 2.8 $NaHCO_3$, 1 $MgCl_2$, 1.5 $CaCl_2$, 15 HEPES, 0.06 $MgSO_4$, pH 7.3 with 10 M NaOH and was kept as a 5× stock solution and sterile-filtered before use.

2.2. Methods

2.2.1. U937 cell culture and preparation. U937 cells were maintained in RPMI 1640 supplemented with 10% FCS at a density of $1–5\times10^5$ cells/ml. For F-actin measurements cells were serum-starved at a density of 5×10^5 cells/ml for 14 h in RPMI supplemented with 0.1% w/v fatty acid free BSA.

2.2.2. Measurement of F-actin content. Serum-starved U937 cells were washed three times and resuspended in U937 balanced salts to a final density of 5×10^5 cells/ml. Cells were preincubated in LP3 tubes (180 μl) for 3 min at 37°C in a water bath. Agonist or vehicle (20 μl) was added at time 0 with gentle vortexing and at timed intervals incubations were quenched by addition of 3.5 ml of isosmotic 4% paraformaldehyde. Assay tubes were placed on ice for 20 min prior to centrifugation to pellet cells, aspiration and resuspension in 4 ml of PBS. After 3 further washes with PBS, samples were aspirated and resuspended in 100 μl PBS to which 100 μl of 10 μg/ml FITC-phalloidin was subsequently added. The tubes were vortexed and incubated in a dark room for 30 min. Samples were washed once with 4 ml PBS before centrifugation, aspiration and resuspension in a final volume of 750 μl PBS. FITC-phalloidin content was determined with a fluorescence activated cell sorter (FACScan) reading at 525 nm using consort 30 software. Measurements presented are modal shifts (means ± S.E.M.) in the fluorescence peak (10 000 events per sample)

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at 525 nm and normalized relative to controls from n separate experiments.

3. Results

3.1. Insulin and ATP stimulate an increase in F-actin content in U937 cells

Insulin and ATP stimulate PtdIns(3,4,5)P_3 production in U937 cells [13], and consequently the effect of these mitogens upon cellular F-actin content was assessed by FACScan quantification of FITC-phalloidin binding in permeabilized cells. Fig. 1 shows that 10 μg/ml insulin (a maximal effective dose of insulin for both receptor occupation and PtdIns(3,4,5)P_3 accumulation; F.T.C. unpublished data) evoked a slow and transient 30 ± 8% increase in F-actin content within 1 min of addition ($n = 6$), which was reversed in the continuous presence of agonist. ATP (100 μM) evoked a rapid 133 ± 6% increase in F-actin content within 5 s of addition ($n = 6$) which was sustained for the 2 min of agonist addition. A dose-response relation for ATP is shown in Fig. 2, where maximal responses to ATP are elicited at 100 μM with an EC_{50} of 200 nM. The difference in the time courses, amplitude and kinetics of the two responses suggests that distinct second messenger pathways may mediate the responses to insulin and ATP. Fig. 3 shows that the addition of 200 nM thapsigargin, an inhibitor of the endoplasmic reticular Ca^{2+}-ATPase, evokes a transient 23 ± 4% increase in F-actin content within 15 s of addition ($n = 3$). However, 1 μM TPA, a non-specific activator of protein kinase C (PKC), has no effect upon F-actin content in a 5 min time course ($n = 3$, Fig. 3). These data suggest that increases in free Ca^{2+}, and not PKC activation, may mediate the action of ATP.

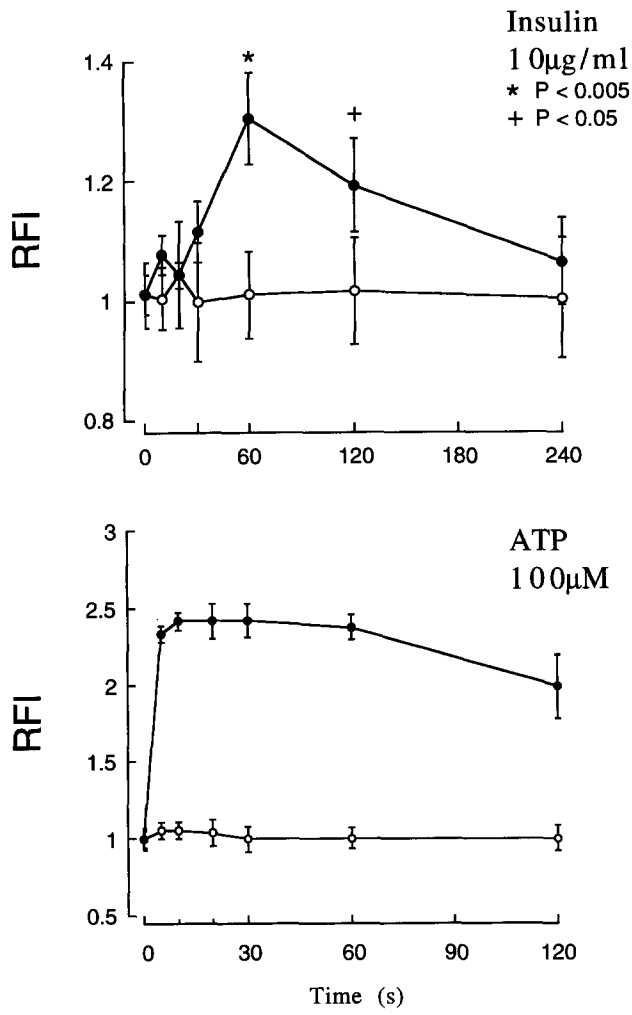

Fig. 1. Insulin and ATP stimulate an increase in F-actin content in U937 cells. The two graphs show the effect of agonist addition upon relative fluorescence intensity (RFI) of FITC-phalloidin binding, a specific marker for F-actin, in permeabilised U937 cells. (Top) At 0 time 10 μg/ml insulin (●) or control salts (○) were added to serum-starved U937 cells and incubations were quenched at timed intervals with 4% paraformaldehyde. (Bottom) The changes in F-actin content in U937 cells stimulated with 100 μM ATP (●) or control salts (○) are shown. Results are expressed as means ± S.E.M. from 6 experiments.

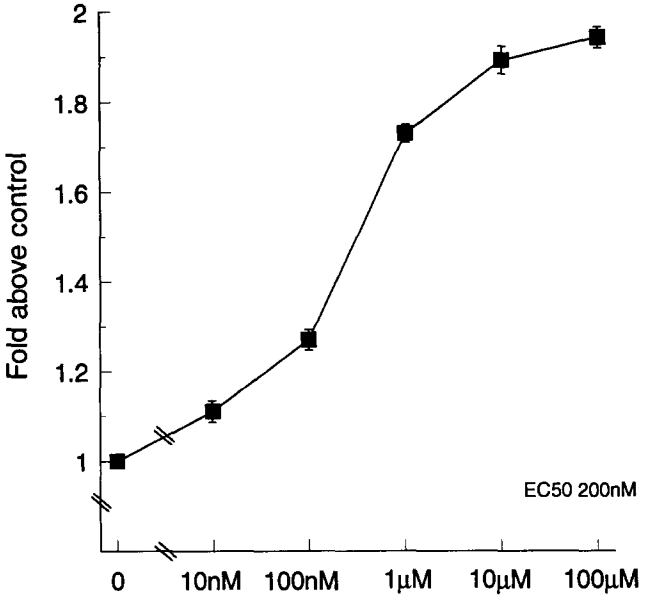

Fig. 2. ATP stimulates a dose-dependent increase in F-actin content. Changes in relative fluorescence intensity (RFI) 10 s after ATP addition are plotted as a function of ATP concentration on a logarithmic scale. Data are expressed as means ± S.E.M. from 3–6 experiments. The half-maximally effective dose (EC_{50}) is given.

3.2. The response to ATP is inhibited by BAPTA and wortmannin

To test the hypothesis that increases in free Ca^{2+} mediate the response to ATP in U937 cells, cell suspensions were preincubated for 30 min with either 80 μM BAPTA-AM [14], a membrane-permeant Ca^{2+} chelator, or DMSO vehicle (0.1% v/v) at 37°C after which time courses following ATP addition or control salts were then performed. The response to ATP was not substantially altered by preincubation with DMSO (Fig. 4), although BAPTA preincubation abolished the ATP response ($n = 4$). As ATP has been shown to stimulate increases in PtdIns(3,4,5)P_3 levels in U937 cells [13], the effect of a 150 s preincubation with 800 nM wortmannin upon submaximal concentrations of ATP (100 nM) were tested. These relatively high concentrations of wortmannin were shown to be required to completely inhibit ATP-stimulated PtdIns(3,4,5)P_3 accumulation. Fig. 4 shows that wortmannin inhibits the sustained, but not the early component of the increase in F-actin content in response to 100 nM ATP

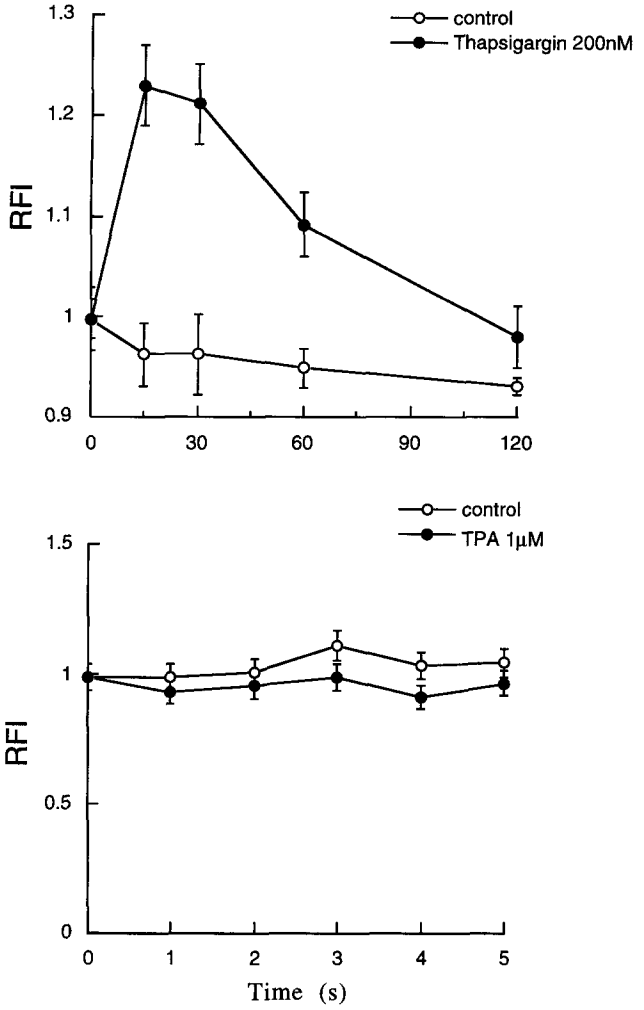

Fig. 3. Thapsigargin, but not phorbol ester, stimulates an increase in F-actin content. (Top) Effect of addition of 200 nM thapsigargin (●) or vehicle (○) at 0 time upon relative fluorescence intensity (RFI) in serum-starved U937 cells over a 120 s time course. (Bottom) Effect of addition of 1 μM TPA (●) or vehicle (○) upon F-actin content over a 5 min time course. Results are expressed as means ± S.E.M. from 3 separate experiments.

($n = 4$), suggesting that the ATP receptor response is mediated by separable Ca^{2+} and PtdIns(3,4,5)P_3-mediated signaling pathways.

3.3. *Wortmannin inhibits both ATP and insulin-evoked increases in F-actin content*

The effects of wortmannin upon agonist-stimulated increases in F-actin content 1 min after addition of insulin or ATP is shown in Fig. 5. The response to 100 nM ATP was significantly inhibited ($P < 0.01$) at 1 min following a 150 s preincubation with 800 nM, but not 100 nM wortmannin ($n = 6$), whereas the response to 10 μg/ml insulin at 1 min was blocked by both 100 and 800 nM wortmannin ($n = 6$). The dose dependency observed here is similar to that reported previously for the protein tyrosine kinase and G-protein receptor-coupled isoforms of PI3K [13].

4. Discussion and conclusions

Growth-factor regulated signaling pathways have been implicated in the control of a number of actin-dependent cytoskeletal changes, including neurite outgrowth, stress fibre formation, membrane ruffling and microspike formation. The signalling pathways leading to actin-dependent changes in cell motility are steadily being unravelled. Membrane ruffling evoked by PDGF and insulin in Swiss 3T3 cells [6] is mediated through activation of the ras-related GTPase rac [5] in a PI3K-dependent manner [7], whereas formation of stress fibres and focal adhesions elicited by lysophosphatidic acid and bombesin in these cells is mediated via activation of the GTPase rho [15] in a PI3K-independent manner [6]. Wortmannin has proved to be a very useful inhibitor of PI3K due to its cell permeability and potency, however, it is not completely specific and particularly at higher doses (greater than 200 nM) other enzymes can be inhibited, e.g. myosin light chain kinase and DNA-dependent protein kinase [16]. This means that we cannot claim to have shown that ATP-stimulated F-actin accumulation is PI3K-dependent. However, it appears that ATP and insulin stimulate

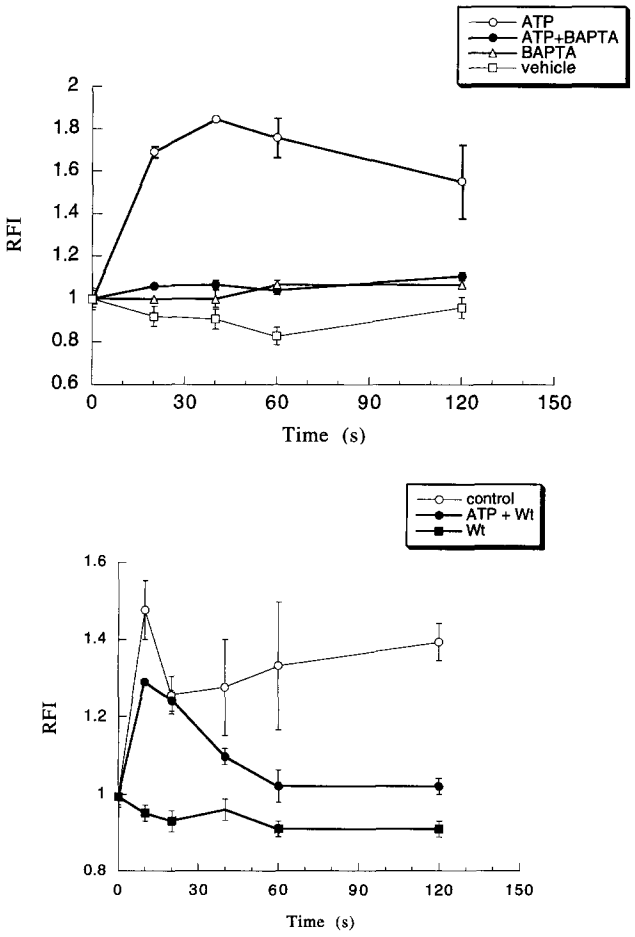

Fig. 4. Increases in F-actin content are inhibited by preincubation with BAPTA or wortmannin. (Top) Effect of addition of 100 nM ATP (○), 800 nM wortmannin (■) or 100 nM ATP + 800 nM wortmannin (●) upon relative fluorescence intensity (RFI) in U937 cells. (Lower) Effect of BAPTA preincubation upon changes in F-actin content stimulated by 100 μM ATP. Time-dependent changes in F-actin content following 30 min preinbations with 80 μM BAPTA (△), DMSO vehicle (□), 100 μM ATP (○) or 80 μM BAPTA + 100 μM ATP (●). Data are expressed as means ± S.E.M. from 4 separate experiments.

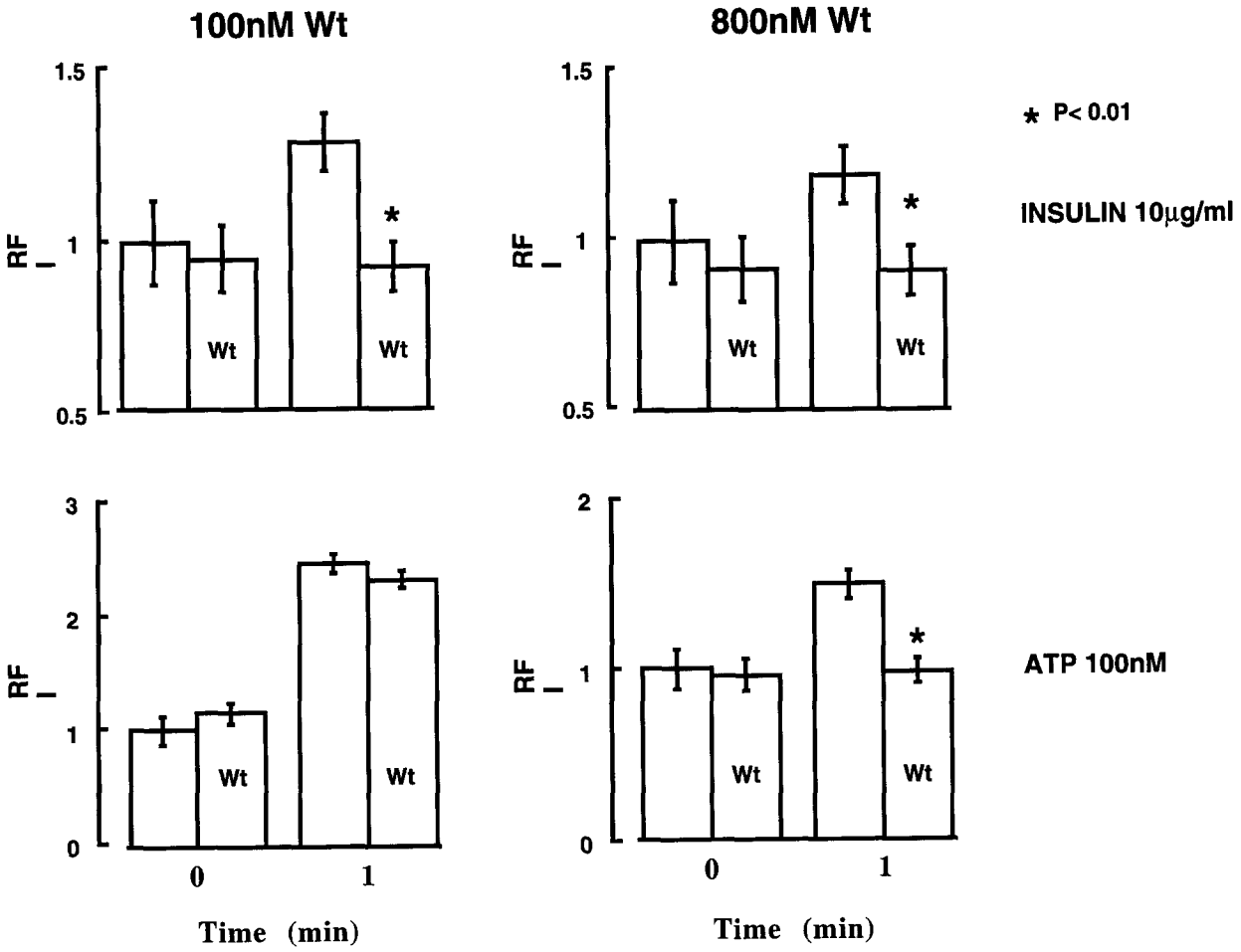

Fig. 5. F-actin polymerization stimulated by both ATP and insulin is inhibited by wortmannin. Histograms showing changes in relative fluorescence intensity (RFI) before (0 min) and after (1 min) addition of either 10 μg/ml insulin (top row) or 100 nM ATP (bottom row) in the absence (hollow bars) or presence (Wt) of either 100 nM (left column) or 800 nM (right column) wortmannin. Results are presented as means ± S.E.M. from 6 separate paired experiments and asterisks indicate significance ($P < 0.01$) calculated by Student's paired t-test.

PtdIns(3,4,5)P_3 accumulation in U937 cells by activating distinct PI3Ks [13]. Insulin activates a classical, p85/p110, form of PI3K, which can be completely inhibited by 100 nM wortmannin in vitro. Hence, our observation that 100 nM wortmannin blocks both insulin-stimulated PtdIns(3,4,5)P_5 accumulation and F-actin accumulation in intact U937 cells is consistent with a role for PI3K in this process. ATP stimulates PtdIns(3,4,5)P_3 accumulation in U937 cells, probably by activating a G-protein βγ-subunit sensitive form of PI3K that is significantly less sensitive to wortmannin in vitro. This correlates with the lower wortmannin sensitivity of ATP-stimulated PtdIns(3,4,5)P_3 accumulation in intact U937 cells (complete inhibition by 800 nM wortmannin) [13]. Hence, our observation (above) that 800 nM wortmannin is required to inhibit the late component of ATP-stimulated F-actin accumulation is consistent with this; and indeed in many ways the overall correlation between inhibition of both insulin and ATP stimulated PI3Ks and F-actin accumulation strengthens the concept of an underlying causal relationship. However, it is clear that the above data could also be explained by postulating that, e.g. a myosin light chain kinase is responsible for late-phase F-actin accumulation in ATP-stimulated U937 cells.

Our data are consistent with a distinct signalling pathway, involving ATP-stimulated increases in free Ca^{2+} and PI3K activity, in the regulation of actin polymerization and arrangement. Interestingly, this appears to parallel the biphasic phosphorylation of pleckstrin stimulated by thrombin [17]. The early phase of phosphorylation is calcium dependent, the later phase being calcium-independent and probably PI3K-dependent. Perhaps this suggests pleckstrin could be a common target of calcium and PI3K-dependent processes and that pleckstrin phosphorylation may be a key step in the agonist-stimulated F-actin responses we have measured.

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