Sorbitol activates atypical protein kinase C and GLUT4 glucose transporter translocation/glucose transport through proline-rich tyrosine kinase-2, the extracellular signal-regulated kinase pathway and phospholipase D

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Sorbitol, 'osmotic stress', stimulates GLUT4 glucose transporter translocation to the plasma membrane and glucose transport by a phosphatidylinositol (PI) 3-kinase-independent mechanism that reportedly involves non-receptor proline-rich tyrosine kinase-2 (PYK2) but subsequent events are obscure. In the present study, we found that extracellular signal-regulated kinase (ERK) pathway components, growth-factor-receptor-bound-2 protein, son of sevenless (SOS), RAS, RAF and mitogen-activated protein (MAP) kinase/ERK kinase, MEK(-1), operating downstream of PYK2, were required for sorbitol-stimulated GLUT4 translocation/glucose transport in rat adipocytes, L6 myotubes and 3T3/L1 adipocytes. Furthermore, sorbitol activated atypical protein kinase C (aPKC) through a similar mechanism depending on the PYK2/ERK pathway, independent of PI 3-kinase and its downstream effector, 3-phosphoinositide-dependent protein kinase-1 (PDK-1). Like PYK2/ERK pathway components, aPKCs were required for sorbitol-stimulated GLUT4 translocation/glucose transport. Interestingly, sorbitol stimulated increases in phospholipase D (PLD) activity and generation of phosphatidic acid (PA), which directly activated aPKCs. As with aPKCs and glucose transport, sorbitol-stimulated PLD activity was dependent on the ERK pathway. Moreover, PLD-generated PA was required for sorbitol-induced activation of aPKCs and GLUT4 translocation/glucose transport. Our findings suggest that sorbitol sequentially activates PYK2, the ERK pathway and PLD, thereby increasing PA, which activates aPKCs and GLUT4 translocation. This mechanism contrasts with that of insulin, which primarily uses PI 3-kinase, D3-PO_4 polyphosphoinositides and PDK-1 to activate aPKCs.

Key words: adipocytes, insulin, 3-kinase, myocytes, phosphatidic acid, phosphatidylinositol.

INTRODUCTION

Like insulin, the non-metabolizable carbohydrate, sorbitol, postulated to act through an 'osmotic stress' sensor, stimulates the translocation of the GLUT4 glucose transporter to the plasma membrane and thereby increases glucose transport in 3T3/L1 adipocytes [1] and muscle cells [2]. In this regard, insulin-stimulated GLUT4 translocation is thought to be effected through activation of phosphatidylinositol (PI) 3-kinase and subsequent increases in D3-PO_4 polyphosphoinositides, in particular, PI 3,4,5-trisphosphate (PIP_3), which, in conjunction with increases in the action of 3-phosphoinositide-dependent protein kinase-1 (PDK-1), activate atypical protein kinase C (aPKC) isoforms, ζ, λ and ι [3–6] and protein kinase B [7–10]. In contrast, sorbitol-stimulated GLUT4 translocation/glucose transport occurs through a mechanism that is largely independent of PI 3-kinase and protein kinase B [1]. However, other than the fact that sorbitol phosphorylates/activates the non-receptor tyrosine kinase, proline-rich tyrosine kinase-2 (PYK2) in 3T3/L1 adipocytes [1], there is relatively little insight into factors that are operative during sorbitol-stimulated GLUT4 translocation in these and other cells.

PYK2 is known [11] to activate mitogen-activated protein (MAP) kinases, including the extracellular signal-regulated kinases 1 and 2 (ERK1/2). Furthermore, the activation of the RAS/ERK pathway, in certain instances, is known to increase glucose transport [12,13]. Therefore, we examined the possibility of sorbitol utilizing the PYK2/RAS/ERK pathway to stimulate GLUT4 translocation/glucose transport in several cell types. Interestingly, we found that not only did sorbitol utilize the PYK2/ERK pathway to stimulate GLUT4 translocation/glucose transport, but it also effected PYK2/ERK-dependent activation of aPKCs. The mechanism was independent of PI 3-kinase and PDK-1, but dependent on the activation of phospholipase D (PLD), which through increases in phosphatidic acid (PA) is thought to activate aPKCs [14,15].

EXPERIMENTAL

Rat adipocyte preparation and incubation conditions

As described previously [4,16–20], rat adipocytes were prepared by collagenase digestion of epididymal fat pads, and either used directly or transfected and cultured overnight as described below. In either case, the cells were finally incubated in glucose-free Krebs Ringer phosphate (KRP) medium containing 1% BSA first for 15 min (or 45 min when the cell-permeable myristoylated PKC-ζ pseudosubstrate was used to allow sufficient time for

Abbreviations used: (a)PKC, (atypical) protein kinase C; ERK, extracellular signal-regulated kinase; PYK2, proline-rich tyrosine kinase-2; DOG, deoxyglucose; Grb2, growth-factor-receptor-bound-2 protein; SOS, son of sevenless; PI, phosphatidylinositol; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; KRP, Krebs Ringer phosphate; KI, kinase inactive; HA, haemagglutinin antigen; PLD, phospholipase D; MAP, mitogen-activated kinase; MEK(-1), mitogen-activated protein (MAP) kinase/ERK kinase; PA, phosphatidic acid.

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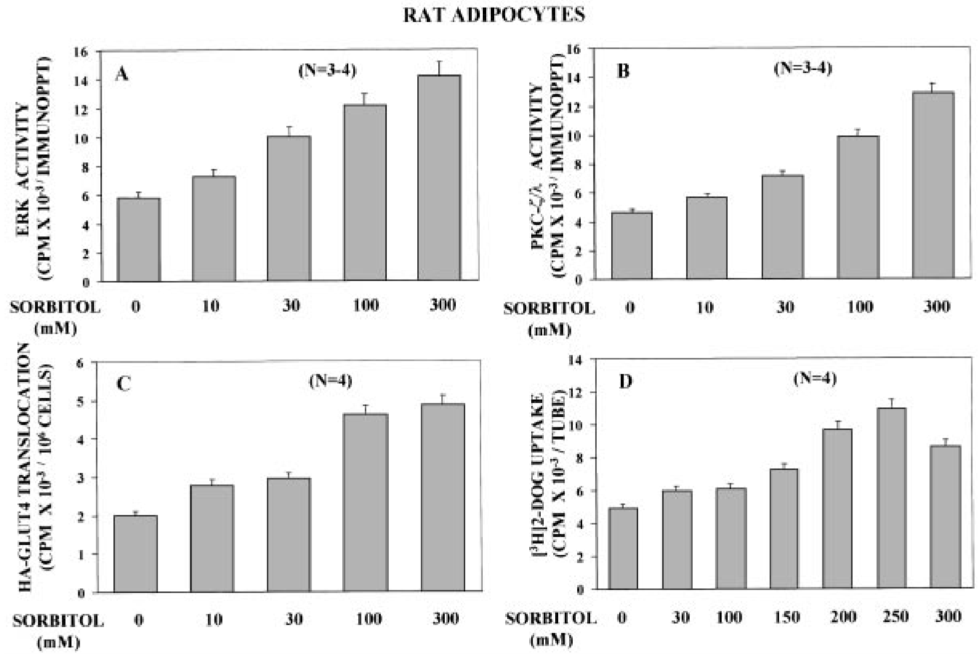

Figure 1 Dose-dependent effects of sorbitol

(**A**) ERK activity, (**B**) PKC-ζ/λ activity, (**C**) HA-GLUT4 translocation to the plasma membrane and (**D**) [^{3}H]2-DOG uptake during sorbitol treatment of rat adipocytes. Adipocytes were treated with different concentrations of sorbitol for 15 min in enzyme activation studies, or for 30 min in GLUT4 translocation studies, following which, cell lysates were examined for immunoprecipitable ERK or aPKC activity, or translocation of HA-GLUT4 to the plasma membrane, or [^{3}H]2-DOG uptake was measured in intact cells.

cellular uptake [4]), with or without inhibitors at concentrations as indicated [wortmannin (Sigma), genistein (Calbiochem), PD98059 (Alexis), UO126 (Promega); dantrolene (Alexis), and myristoylated PKC-ζ pseudosubstrate (Biosource); SB202190 (Tocris)], and then incubated for specified times with or without sorbitol, insulin, PLD (from *Streptomyces* species; Sigma) or other agonists for studies of [^{3}H]2-deoxyglucose (DOG) uptake, translocation of transiently transfected epitope-tagged GLUT4 glucose transporters to the plasma membrane, PYK2 activation, ERK activation or PKC-ζ/λ activation, as described below.

3T3/L1 adipocyte and L6 myotube preparation and incubation conditions

3T3/L1 adipocytes [3] and L6 myotubes were cultured, differentiated, serum-starved for 3–4 h, and finally incubated in glucose-free KRP medium with or without inhibitors, sorbitol and/or insulin, as described previously [5,21].

Transfections

Translocation of epitope-tagged GLUT4 glucose transporter was examined by the method described in refs [4,16,17,19]. Rat adipocytes (0.4 ml) were transiently co-transfected by electroporation in an equal volume of Dulbecco's modified Eagle's medium (DMEM) containing 3 μg pCIS2 encoding HA-GLUT4 (where HA is haemagglutinin antigen) with or without 7 μg (or occasionally 14 μg, as noted) plasmid alone (vector) or plasmid [4,16–20] encoding dominant-negative forms of son of sevenless (SOS) 9 (in pSRα; kindly supplied by Dr Masato Kasuga), RAS (in pRSV; kindly supplied by Dr Jane Reusch), Grb2 (growth-factor-receptor-bound-2 protein) (in pCGN), PYK2 (in pRK5), cRAF-1 (in pEF), MAP kinase/ERK kinase (MEK1) (in pCDNA3), PDK-1 (in pCDNA3) or PKC-ζ (in pCDNA3). After transfection, cells were incubated overnight in DMEM containing 5 % BSA, washed, and incubated for specified times in glucose-free KRP medium containing 1 % BSA with or without sorbitol, insulin or other substances as described above. After incubation, translocation of epitope-tagged GLUT4 to the plasma membrane was determined by measurement of the cell surface level of exofacial epitope as detected with mouse monoclonal anti-HA (Covance, Berkeley, CA, U.S.A.) primary antibody and ^{125}I-labelled anti-mouse IgG secondary antibody (Amersham Pharmacia Biotech) as described previously [4,16–19].

For studies of epitope-tagged ERK activation, as described in [18,20], 0.4 ml of rat adipocytes was co-transfected by electroporation with an equal volume of DMEM containing 3 μg pCEP4 encoding HA-ERK2 or MYC-ERK2 (kindly supplied by Dr Melanie Cobb), along with 7 μg plasmid encoding dominant-negative forms of PYK2, Grb2, SOS, RAS, cRAF-1 or MEK1 (see above). After overnight incubation in DMEM/BSA medium, cells were washed and incubated for specified times in glucose-free KRP medium containing 1 % BSA, with or without sorbitol or other substances, following which, HA-ERK2 or MYC-ERK2 was precipitated with mouse monoclonal anti-HA (Covance) or anti-MYC (Upstate Biotechnologies Inc., Lake Placid, NY, U.S.A.) antibodies and assayed as described below.

For studies of epitope-tagged PKC-ζ activation, 0.4 ml of rat adipocytes was co-transfected by electroporation with an equal volume of DMEM containing 1 μg pCMV5 encoding FLAG-PKC-ζ (kindly supplied by Dr Alex Toker [16]) or pCDNA3 encoding HA-PKC-ζ, along with 7 μg plasmid encoding dominant-negative forms of PYK2, Grb2, SOS, RAS, cRAF-1 or MEK1 (see above). After overnight incubation in DMEM/BSA medium, cells were washed and incubated for specified times in glucose-free KRP medium containing 1 % BSA, with or without sorbitol or other substances, following which, HA-PKC-ζ or FLAG-PKC-ζ was precipitated with mouse monoclonal anti-HA (Covance) or anti-FLAG (Sigma, St Louis, MO, U.S.A.) antibodies and assayed as described below.

Adenoviral gene transfer studies

Adenoviruses encoding kinase-inactive (KI) forms of PKC-ζ and PDK-1 were constructed using plasmid cDNA inserts [4,16–19] and Adeno-X Expression kits obtained from Clontech. 3T3/L1 adipocytes and L6 myotubes were infected with 10 MOI adenovirus alone or adenovirus encoding KI-PKC-ζ or KI-PDK-1 [21]. After 48 h of incubation, cells were incubated in glucose-free KRP medium with or without sorbitol, insulin or other substances for studies of PKC-ζ activation or glucose transport. Note that it was not possible to conduct adenoviral gene transfer studies of glucose transport in rat adipocytes as they started leaking with adenoviral infection.

ERK activation

Immunoprecipitable ERK activity was measured as described previously [18,20]. In some cases, ERK activation was assessed by Western analysis using phospho-ERK1/2 antiserum (Santa Cruz Biotechnologies) after resolution of p42 and p44 ERK by SDS/PAGE.

Atypical PKC activation

PKC-ζ/λ activity was measured as described previously [3–6,16,17,19]. In brief, aPKCs were immunoprecipitated from salt/detergent-treated cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Inc.) that recognizes the C-termini of both PKC-ζ and PKC-λ for studies of total endogenous aPKC activity, or with mouse monoclonal anti-FLAG (Sigma) or anti-HA (Covance) antibodies for studies of epitope-tagged PKC-ζ activity. Precipitates were collected on Sepharose–AG beads (Santa Cruz Biotechnologies) and incubated for 8 min at 30 °C in 100 μl buffer containing 50 mM Tris/HCl (pH 7.5), 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM PMSF, 4 μg phosphatidylserine (Sigma), 50 μM [γ-^{32}P]ATP (NEN Life Science Products), 5 mm $MgCl_2$ and, as substrate, 40 μM serine analogue of the PKC-ϵ pseudosubstrate (BioSource), a preferred substrate for aPKCs. After incubation, ^{32}P-labelled substrate was trapped on P-81 filter paper and counted.

PYK2 activation

PYK2 activation was assessed by Western analysis for phosphorylation of Y402, the autophosphorylation site, and Y881, the Grb2-interacting site [11,18], using phosphopeptide-specific antisera obtained from Biosource, after resolution of 120 kDa PYK2 by SDS/PAGE, as described previously [18].

PLD activation

PLD was assayed [22] by generation of ^{3}H-labelled phosphatidylethanol or phosphatidylbutanol (results were essentially the same) in cells prelabelled by overnight incubation with [^{3}H]oleic acid (NEN Life Science Products) in DMEM, followed by washing and incubation for 15 min in glucose-free KRP medium containing 1.7% ethanol or n-butanol. These primary alcohols substitute for water during PLD-mediated hydrolysis of phosphatidylcholine and perhaps other phospholipids to yield phosphatidylethanol or phosphatidylbutanol, instead of PA.

Glucose transport

Cells were incubated for 30 min in glucose-free medium with or without sorbitol or insulin, following which, uptake of [^{3}H]2-DOG was measured for 1 min in rat adipocytes [4,16,17], and for 5 min in 3T3/L1 adipocytes [3] and L6 myotubes [5,21]. The results in the figures are expressed as mean ± S.E.M. of the number of determinations.

RESULTS

Studies in rat adipocytes

Sorbitol activates ERK, PKC-ζ/λ and GLUT4 translocation/glucose transport

As seen in Figure 1, sorbitol stimulated dose-related increases in ERK activity, PKC-ζ/λ activity, translocation of HA-GLUT4 glucose transporter to the plasma membrane and [^{3}H]2-DOG uptake in rat adipocytes. Subsequently, [^{3}H]2-DOG uptake

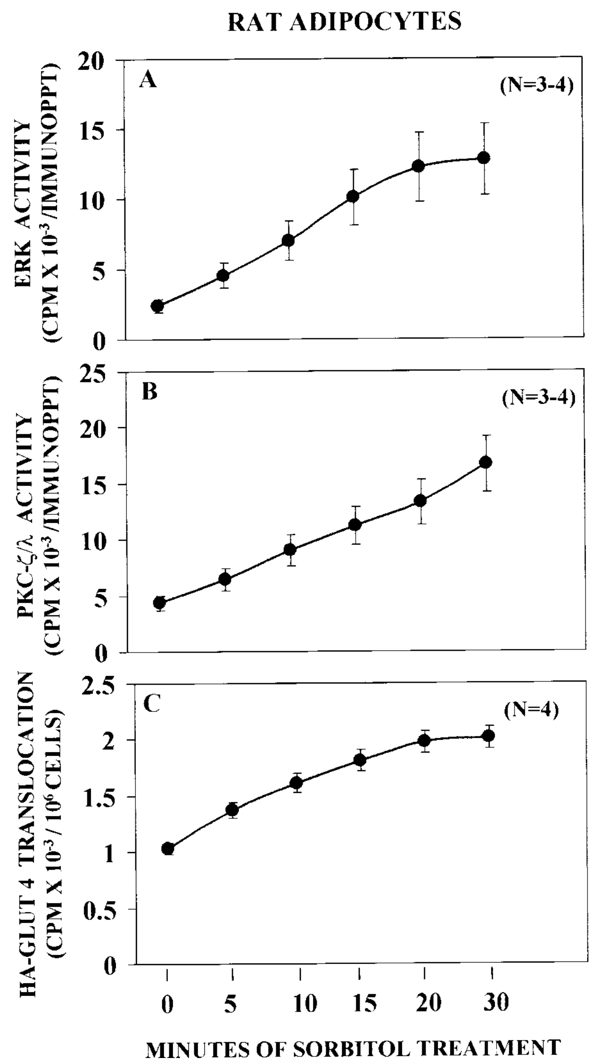

Figure 2 Sorbitol-induced increases in activity as a function of time

(**A**) ERK activity, (**B**) PKC-ζ/λ activity and (**C**) HA-GLUT4 translocation to the plasma membrane during sorbitol treatment of rat adipocytes. Adipocytes were treated for specified times with 300 mM sorbitol, following which, cell lysates were examined for immunoprecipitable ERK or aPKC activity, or translocation of HA-GLUT4 to the plasma membrane.

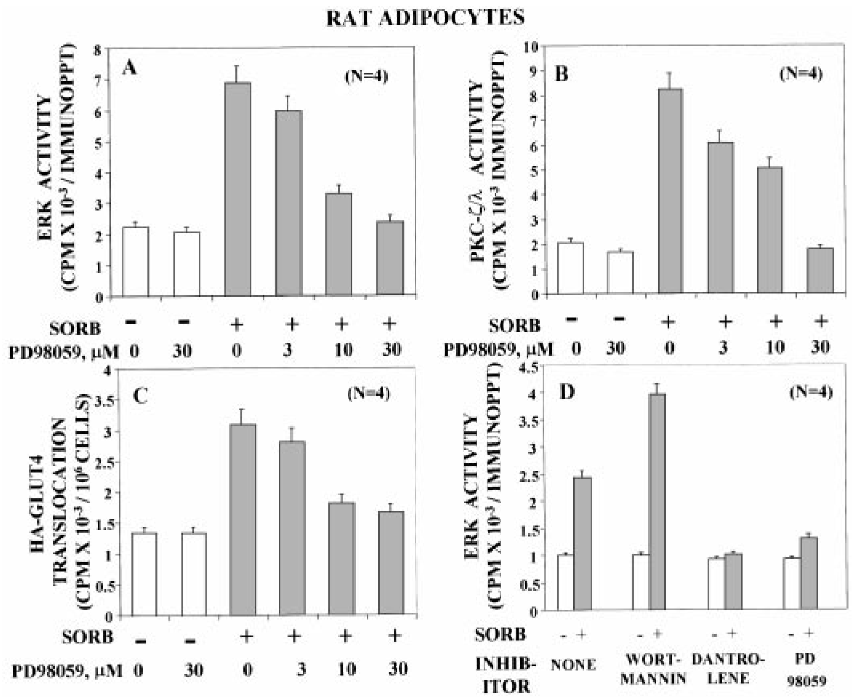

Figure 3 Effects of inhibitors of MEK1 [25 μM PD98059 in (D) and concentrations as indicated in (A)–(C), PI 3-kinase [100 nM wortmannin in (D)], and PYK2 [25 μM dantrolene in (D)] on sorbitol-induced activity in rat adipocytes

(**A, D**) ERK activity, (**B**) PKC-ζ/λ activity and (**C**) HA-GLUT4 translocation to the plasma membrane. Adipocytes were first treated for 15 min with inhibitors as indicated, and then incubated with or without 300 mM sorbitol (SORB) for 15 min in enzyme activation studies, or for 30 min in GLUT4 translocation studies, following which, cell lysates were examined for immunoprecipitable ERK or aPKC activity, or translocation of HA-GLUT4 to the plasma membrane.

tended to decrease at higher sorbitol concentrations, most likely due to weak binding of sorbitol to glucose-binding sites on glucose transporters and therefore competition with 2-DOG, or, perhaps less likely, as a result of alterations in factors that may be required for glucose transport, in addition to GLUT4 translocation. Because of this secondary diminution in [^{3}H]2-DOG uptake, subsequent studies in rat adipocytes focused upon alterations in HA-GLUT4 translocation, which were maximal or near maximal at 300 mM sorbitol. Time courses for sorbitol-induced increases in activity of ERK and PKC-ζ/λ and HA-GLUT4 translocation are shown in Figure 2.

Effects of inhibitors of signalling factors on sorbitol-induced activation of ERK, PKC-ζ/λ and GLUT4 translocation

PD98059, a selective inhibitor of MEK1 the major activator of ERK, was used in initial studies to determine the ERK pathway during sorbitol action. As seen in Figures 3(A)–3(C), sorbitol-induced increases not only in ERK activity (as expected), but also (more interestingly) in PKC-ζ/λ activity and HA-GLUT4 translocation, were each progressively inhibited by increasing concentrations of PD98059. These inhibitory effects of PD98059 suggested that MEK1 was required for sorbitol-induced activation of ERK, PKC-ζ/λ and HA-GLUT4 translocation. Furthermore they increased the possibility that ERK may be required for activation of PKC-ζ/λ, which, in turn, in view of its apparent involvement in insulin action [3–6,16,17,19,21], may be required for sorbitol-stimulated HA-GLUT4 translocation. Although not shown, genistein inhibited each of these increases, suggesting, as expected [1], a requirement for a tyrosine kinase in sorbitol action. Also, unlike the MEK1 inhibitor, PD98059, the p38MAP kinase inhibitor, SB202190, did not inhibit sorbitol-induced increases in HA-GLUT4 translocation.

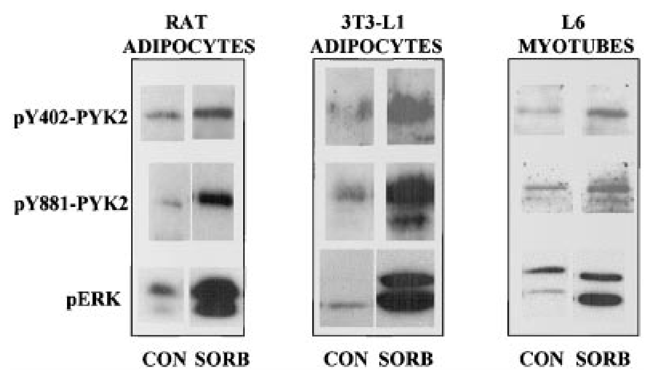

Figure 4 Effects of sorbitol on phosphorylation/activation of PYK2 and ERK in rat adipocytes, 3T3/L1 adipocytes and L6 myotubes

Cells were treated for 15 min with or without (CON) 300 mM sorbitol (SORB), following which, cell lysates were subjected to SDS/PAGE and Western analysis, using phosphopeptide-specific antibodies.

Whereas the activation of ERK in rat adipocytes by insulin requires PI 3-kinase [20], sorbitol-induced increases in ERK were not inhibited (and, in some, but not all cases, for uncertain reasons, were stimulated) by the PI 3-kinase inhibitor, wortmannin (Figure 3D). Since sorbitol is known to stimulate tyrosine autophosphorylation (i.e. activation) of the non-receptor tyro-

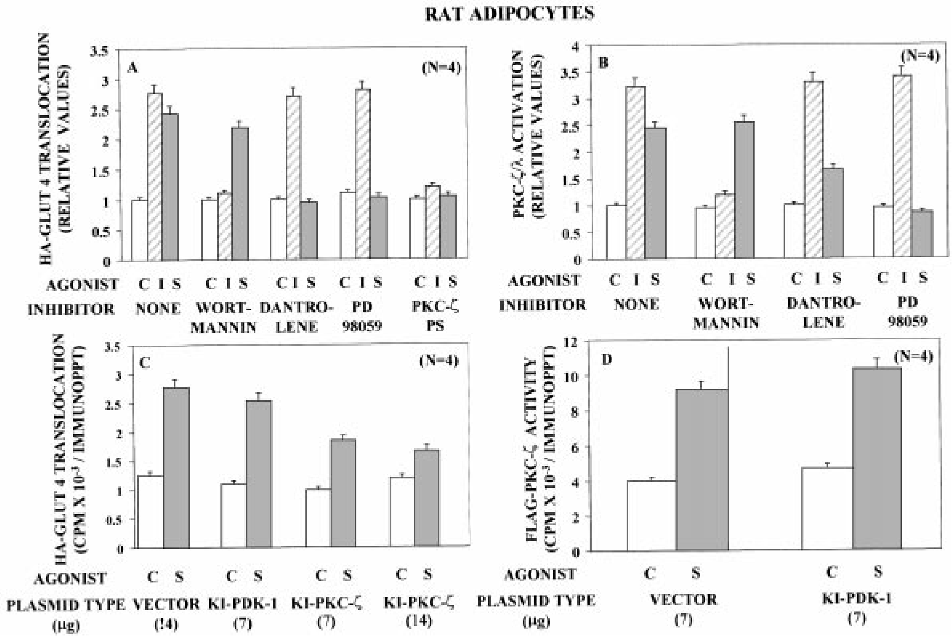

Figure 5 Effects of inhibitors of MEK1 (25 μM PD98059), PI 3-kinase (100 nM wortmannin), PYK2 (25 μM dantrolene) and aPKCs (50 μM cell-permeable myristoylated PKC-ζ pseudosubstrate or micrograms of plasmid DNA encoding KI-PKC-ζ) on sorbitol-induced activity in rat adipocytes as indicated

(**A, C**) HA-GLUT4 translocation to the plasma membrane and (**B**) total cellular PKC-ζ/λ activity or (**D**) FLAG-tagged PKC-ζ in rat adipocytes. Adipocytes were first incubated for 24 h with plasmid encoding KI-PKC-ζ, or for 45 min with the PKC-ζ pseudosubstrate, or for 15 min with concentrations of other inhibitors indicated. They were then incubated with (C) or without 300 mM sorbitol (S) or 10 nM insulin (I) for 15 min in enzyme activation studies, or for 30 min in HA-GLUT4 translocation studies. Cell lysates were then examined for immunoprecipitable ERK or aPKC activity, or translocation of HA-GLUT4 to the plasma membrane.

sine kinase, PYK2, in 3T3/L1 adipocytes [1], it was of interest to find that dantrolene, an inhibitor of an internal Ca^{2+} pool that is required for PYK2 activation [11], inhibited sorbitol-induced activation of ERK in rat adipocytes (Figure 3D). Moreover, as in 3T3/L1 adipocytes, sorbitol stimulated increases in the phosphorylation of Y402, the autophosphorylation site, and Y881, the Grb2-interacting site of PYK2 in rat adipocytes (Figure 4).

Similar to findings for ERK activation (Figure 3D), sorbitol-induced activation of both PKC-ζ/λ and HA-GLUT4 translocation was insensitive to the PI 3-kinase inhibitor, wortmannin, but sensitive to inhibition by the PYK2 inhibitor, dantrolene, as well as the MEK1 inhibitor, PD98059 (Figures 5A and 5B). In contrast, insulin-induced activation of PKC-ζ/λ and GLUT4 translocation was inhibited by wortmannin, but not by dantrolene or PD98059 (Figures 5A and 5B). Thus, sorbitol and insulin activated PKC-ζ/λ and GLUT4 translocation by clearly different mechanisms. In this regard, note that insulin effects on the ERK pathway in rat adipocytes are blocked by wortmannin and PD98059 [20], but not by dantrolene [18]; accordingly, insulin and sorbitol activate the ERK pathway by clearly different mechanisms. Also note that insulin does not activate PYK2 (results not shown).

Effects of expression of dominant-negative forms of PYK2, Grb2, SOS, RAS, RAF and MEK1 on sorbitol-induced activation of ERK, PKC-ζ/λ and GLUT4 translocation

The above findings suggested that both PYK2 and the ERK pathway, but not PI 3-kinase, were required for effects of sorbitol on PKC-ζ/λ activation and HA-GLUT4 translocation in rat adipocytes. Further evidence for the involvement of PYK2 and the ERK pathway during sorbitol action was obtained by finding that expression of the non-catalytic fragment of PYK2, PRNK, which serves as a dominant-negative for PYK2-dependent processes [11,18], inhibited sorbitol-induced activation of ERK, aPKCs and HA-GLUT4 translocation (Figure 6). Furthermore, expression of dominant-negative forms of Grb2, SOS, RAS, cRAF-1 and MEK1 largely inhibited sorbitol-induced increases in activation, not only of epitope-tagged forms of co-expressed ERK2 (as would be expected), but also of increases in PKC-ζ activity and HA-GLUT4 translocation (Figures 6A–6C). Along with insensitivity to dantrolene and PD98059 (Figures 5A and 5B), insulin-induced increases in epitope-tagged PKC-ζ activity and GLUT4 translocation were not inhibited by expression of dominant-negative forms of PYK2, Grb2, SOS and RAS (latter data not shown).

Studies on PDK-1 requirements in sorbitol-induced activation of PKC-ζ/λ

In contrast to PKC-ζ/λ activation and translocation of GLUT4 during insulin action in rat adipocytes [19], the expression of KI-PDK-1 had little or no effect on sorbitol-stimulated FLAG-PKC-ζ activation (Figure 5D). These findings were in accordance with the above-described findings indicating that PI 3-kinase, the immediate activator/facilitator of PDK-1, was not required for sorbitol-induced increases in ERK activity, PKC-ζ/λ activity and GLUT4 translocation in rat adipocytes.

Studies on PKC-ζ/λ requirements for sorbitol-stimulated GLUT4 translocation

In view of the fact that neither PI 3-kinase nor PDK-1 was required for effects of sorbitol on PKC-ζ/λ activation and HA-GLUT4 translocation, it was particularly interesting to find that

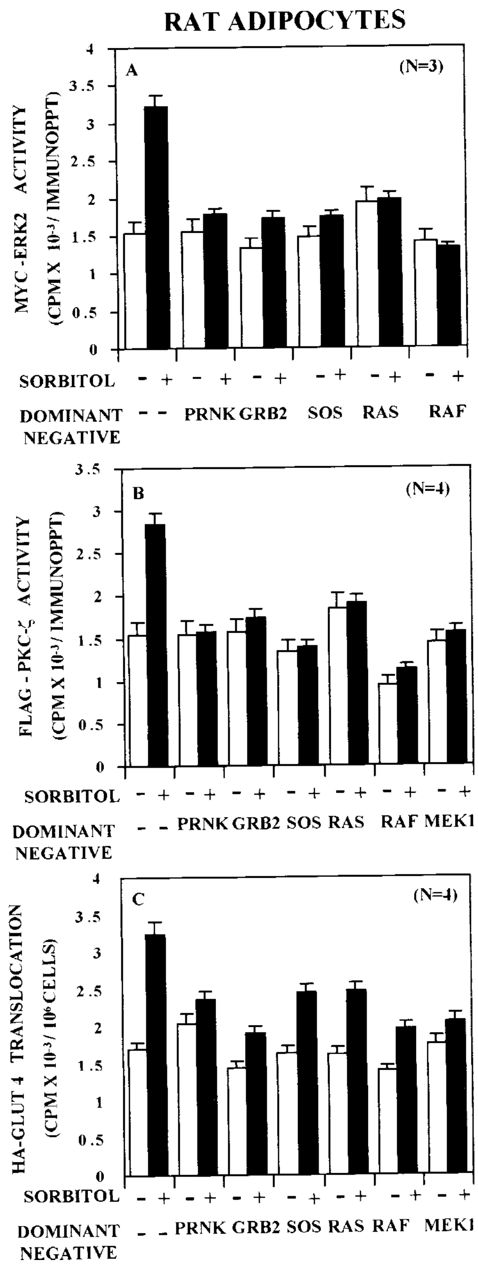

Figure 6 Effects of dominant-negative forms of PYK2 (PRNK), Grb 2, SOS, RAS, c-RAF-1 and MEK1 on activation of epitope-tagged forms of ERK2 and PKC-ζ, and translocation of epitope-tagged GLUT4 to the plasma membrane in rat adipocytes

Adipocytes were transiently co-transfected with plasmids encoding MYC-ERK2, FLAG-PKC-ζ or HA-GLUT4 along with plasmids encoding dominant-negative signalling factors. After overnight incubation, adipocytes were treated with or without 300 mm sorbitol for 15 min in enzyme activation studies, or for 30 min in HA-GLUT4 translocation studies. Cell lysates were examined for immunoprecipitable MYC-ERK2 or FLAG-PKC-ζ activity, or translocation of HA-GLUT4 to the plasma membrane.

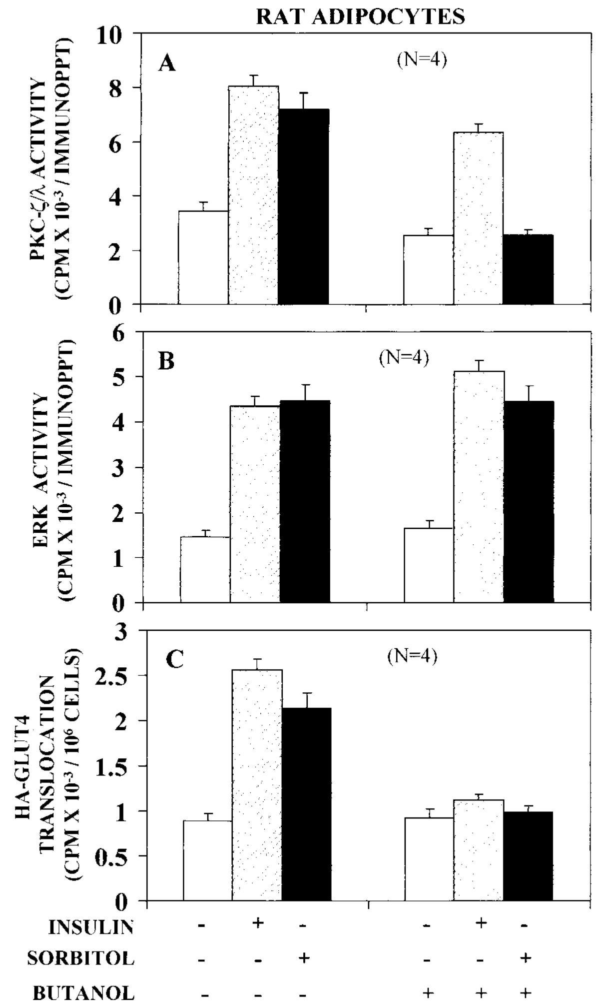

Figure 7 Effects of n-butanol on sorbitol- and insulin-induced increases in rat adipocytes

(**A**) PKC-ζ/λ activity, (**B**) ERK activity and (**C**) HA-GLUT4 translocation. Fresh adipocytes were used (**A, B**) or adipocytes transfected overnight with HA-GLUT4 were used (**C**). All cells were incubated in glucose-free KRP medium first for 5 min with or without 1.5% n-butanol, and then for 15 min (**A**, **B**), or for 30 min (**C**), with or without 300 mm sorbitol or 10 nM insulin, as indicated. After incubation, cell lysates were examined for immunoprecipitable PKC-ζ/λ and ERK activity (**A**, **B**), and cell surface HA-GLUT4 was measured (**C**).

expression of KI-PKC-ζ (Figure 5C) and the cell-permeable myristoylated PKC-ζ pseudosubstrate (Figure 5A) (both of which inhibit insulin-induced activation of PKC-ζ/λ and subsequent GLUT4 translocation/glucose transport [4,16–21]) inhibited sorbitol-induced HA-GLUT4 translocation in rat adipocytes. Thus, it may be surmised that, irrespective of the lack of requirement for PI 3-kinase and PDK-1, PKC-ζ/λ is required for sorbitol-induced activation of the glucose transport system in rat adipocytes.

PLD is required for sorbitol effects on PKC-ζ/λ and GLUT4 translocation

The fact that sorbitol activated PKC-ζ/λ independently of PI 3-kinase prompted us to examine other potential mechanisms. Since PLD-derived PA can directly activate PKC-ζ/λ [14,15], it was of interest to find that n-butanol, which inhibits PLD-dependent PA production by substituting butanol for water during hydrolysis of lipids such as phosphatidylcholine, inhibited sorbitol-induced activation of both PKC-ζ/λ and HA-GLUT4 translocation, but not ERK (Figures 7A–7C). These findings suggested that PLD operated downstream of the ERK pathway, but proximal to PKC-ζ/λ, during sorbitol action. Furthermore, butanol markedly inhibited insulin-induced increases in HA-GLUT4 translocation, but only modestly inhibited insulin-

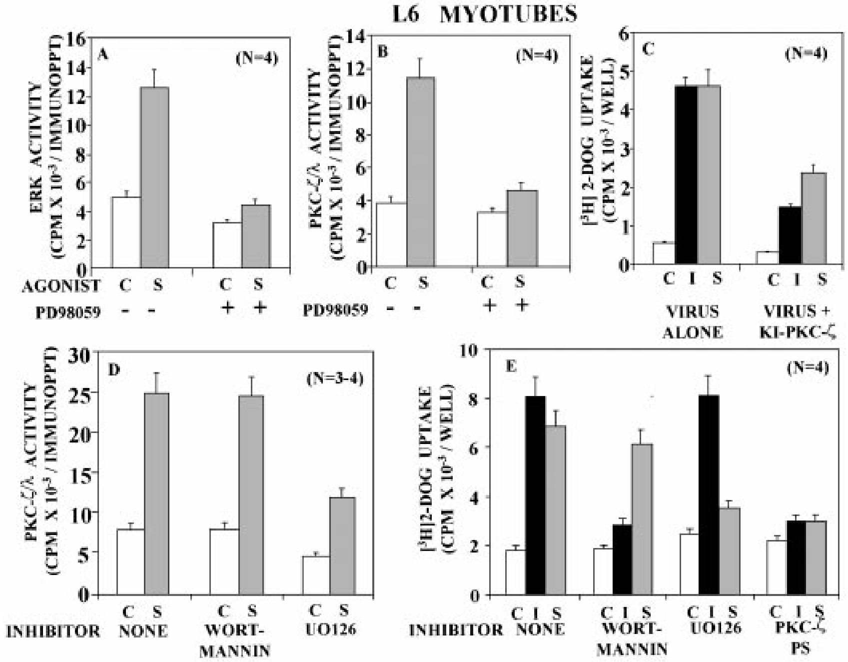

Figure 8 Effects of inhibitors of MEK1 (25 μM PD98059 or 10 μM UO126), PI 3-kinase (100 nM wortmannin), and aPKCs (50 μM cell-permeable myristoylated PKC-ζ pseudosubstrate or 10 MOI adenovirus encoding KI-PKC-ζ) on sorbitol-induced activity in L6 myotubes

(**A**) ERK activity, (**B, D**) total cellular PKC-ζ/λ activity or (**C, E**) [^{3}H]2-DOG uptake. Fully differentiated myotubes were first treated with adenovirus for 48 h (note that adenovirus alone had no effect on glucose transport), or for 45 min with the PKC-ζ pseudosubstrate (PS), or for 15 min with other inhibitors, and then incubated with or without (C) 300 mM sorbitol (S) or 100 nM insulin (I) for 15 min in enzyme activation studies, or for 30 min in glucose transport studies, following which, cell lysates were examined for immunoprecipitable ERK or aPKC activity, or [^{3}H]2-DOG uptake.

induced PKC-ζ/λ activation (approx. 35 % in Figure 7, but this was as little as 10–15 % in some experiments). These findings mimic previously reported findings suggesting that PLD action is required for insulin-stimulated GLUT4 translocation [23], and further suggested that this PLD requirement for glucose transport is not related to PKC-ζ/λ activation during the action of insulin, which, for the most part, activates PKC-ζ/λ through non-PLD signalling pathways, i.e. via PI 3-kinase, PIP_3 and PDK-1.

Studies in L6 myotubes

Analagous to findings in rat adipocytes, sorbitol-induced increases in [^{3}H]2-DOG uptake in L6 myotubes were markedly sensitive to inhibition by MEK1 inhibitors, PD98059 and UO126, but only slightly, sensitive, if at all to the PI 3-kinase inhibitor, wortmannin (Figure 8). Also, in L6 myotubes, as in rat adipocytes, both PYK2 and ERK were activated by sorbitol (Figures 4 and 8) and, based upon MEK1 inhibitor studies, the ERK pathway seems necessary for effects of sorbitol on PKC-ζ/λ activation, as well as on [^{3}H]2-DOG. Similarly, in keeping with findings in rat adipocytes suggesting that PKC-ζ/λ was required for sorbitol-induced increases in glucose transport, both the cell-permeable myristoylated PKC-ζ pseudosubstrate and adenoviral-mediated expression of KI-PKC-ζ inhibited sorbitol-stimulated [^{3}H]2-DOG uptake in L6 myotubes (Figure 8). Note that insulin effects on [^{3}H]2-DOG uptake were markedly sensitive to wortmannin, KI-PKC-ζ and the PKC-ζ pseudosubstrate, but not to the MEK1 inhibitor, UO126 (Figure 8).

Sorbitol was found to activate PLD in L6 myotubes by a mechanism dependent on the ERK pathway (Figure 9A); insulin, on the other hand, activated PLD largely independent of the ERK pathway (results not shown). (Note that it was much easier to conduct such PLD assays involving the separation of labelled phosphatidylbutanol or phosphatidylethanol from other labelled lipids by TLC in these muscle cells, as compared to lipid-laden rat adipocytes; nevertheless, sorbitol increased PLD activity approx. 2-fold in rat adipocytes – data not shown.) As in rat adipocytes, n-butanol markedly inhibited sorbitol-induced increases in PKC-ζ/λ activity, but only mildly inhibited insulin-induced increases in PKC-ζ/λ activity (Figure 9B); this inhibition was only approx. 10 % in Figure 9(B), but was, as in adipocytes, as much as 35 % in other experiments. It may be further noted that (a) the addition of exogenous PLD to L6 myotubes provoked PD98059-insensitive increases in PKC-ζ/λ activity (Figure 9D) and PKC-ζ/λ-dependent increases in [^{3}H]2-DOG uptake (Figure 9C); (b) n-butanol inhibited sorbitol-stimulated [^{3}H]2-DOG uptake (Figure 9E); and (c) PA directly activated PKC-ζ/λ (immunoprecipitated from L6 myotube lysates) when added to *in vitro* assays (Figure 9F). In other studies, PA was found to activate PKC-ζ/λ less effectively than PIP_3. It may be surmised that sorbitol activates PLD, PLD functions downstream rather than upstream of ERK, and the PA generated from the activation of PLD can be fairly expected to activate both PKC-ζ/λ and the glucose transport system.

Studies in 3T3/L1 adipocytes

In addition to increasing PYK2 phosphorylation/activation (Figure 4), sorbitol stimulated increases in ERK phosphorylation (Figure 4), ERK enzyme activation (Figure 10), PKC-λ activation (Figure 10) and [^{3}H]2-DOG uptake (Figure 10) in

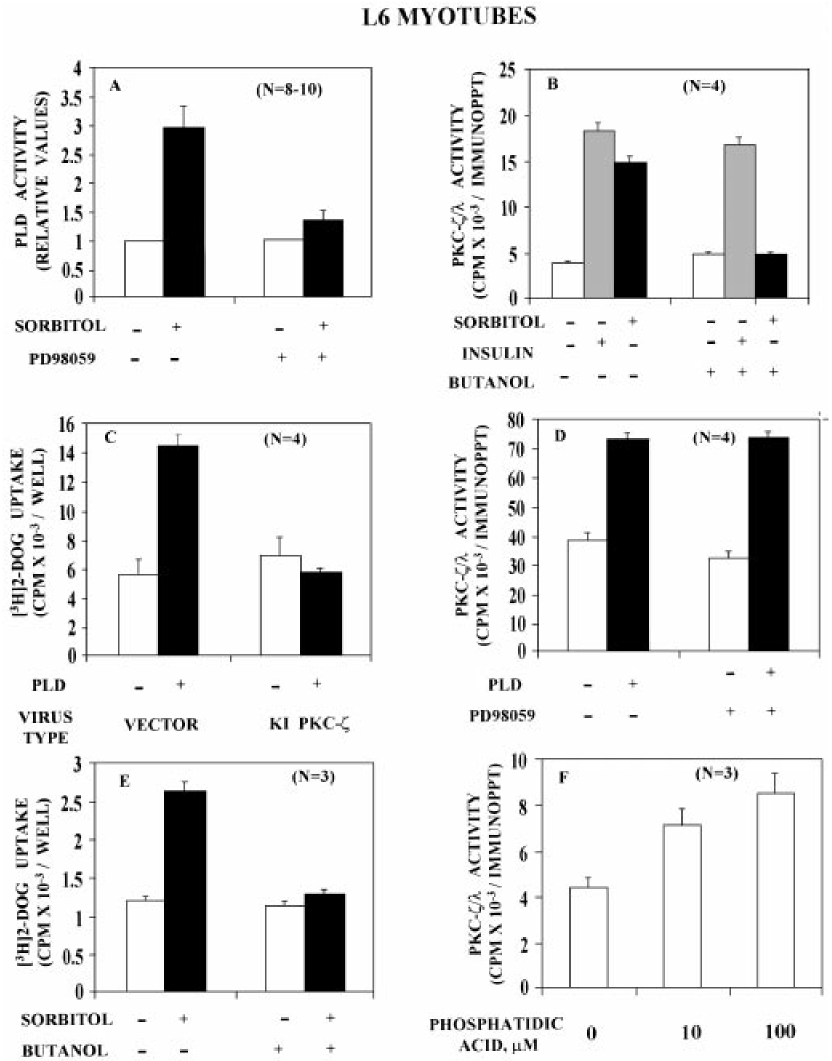

Figure 9 Sorbitol- and insulin-induced activity in L6 myotubes

Effects of (**A**) sorbitol on ERK-dependent PLD activity, (**B**) n-butanol on sorbitol- and insulin-induced increases in PKC-ζ/λ activity, (**C**) PLD on PKC-ζ-dependent [^{3}H]2-DOG uptake, (**D**) PLD on PKC-ζ/λ activity, (**E**) butanol on sorbitol-stimulated [^{3}H]2-DOG uptake, and (**F**) PI on PKC-ζ/λ activity *in vitro* in L6 myotubes. Fully differentiated L6 myotubes were infected with 10 MOI adenovirus or adenovirus encoding KI-PKC-ζ/λ (**C**), or used without viral infection (**A**, **B**, **D**, **E**, **F**). Myotubes were finally incubated in glucose-free KRP medium first for 15 min with or without 35 μM PD98059 (**A**, **D**), or for 5 min with or without 1.7% n-butanol (**B**, **E**). Cells were then treated with or without 300 mm sorbitol, 100 nM insulin or PLD (5 units/ml; from *Streptomyces* species) for 15 min (**A**, **B**, **E**), or for 30 min (**C**, **D**), following which PLD and PKC-ζ/λ activity and [^{3}H]2-DOG uptake were determined. (**F**) PKC-ζ/λ was immunoprecipitated from L6 myotube lysates and assayed in the presence of PA concentrations as indicated.

3T3/L1 adipocytes. As in rat adipocytes and L6 myotubes, sorbitol-stimulated [^{3}H]2-DOG uptake in 3T3/L1 adipocytes was inhibited by MEK1 inhibitors, PD98059 and UO126, and by adenoviral-mediated expression of KI-PKC-ζ, but only slightly, if at all, by wortmannin (Figure 10).

DISCUSSION

Our findings support the previous suggestion [1] that the non-receptor tyrosine kinase PYK2 was required for sorbitol-stimulated GLUT4 translocation/glucose transport in rat adipocytes and L6 myotubes, as well as 3T3/L1 adipocytes, and further suggested that the ERK pathway functioned downstream of PYK2 in this capacity. This requirement for PYK2-dependent ERK activation during sorbitol stimulation contrasts with the lack of requirement for the ERK pathway during insulin-stimulated glucose transport. The reason for this difference is not clear, but note that: (a) PI 3-kinase, PDK-1 and aPKCs, in conjunction with the Grb2/SOS/RAS/RAF/MEK1 pathway, function upstream of ERK ([20] and unpublished work) during insulin action in the cell types studied in this paper; (b) sorbitol effects on ERK are mediated through PYK2 and do not require PI 3-kinase, PDK-1 or aPKCs; (c) unlike sorbitol, insulin neither activates PYK2 nor requires PYK2 for ERK activation [18]; (d) PLD functions largely downstream of the ERK pathway during sorbitol action and largely downstream of PI 3-kinase [22] (but not ERK; unpublished work) during insulin action; and (e) aPKCs function largely downstream of PYK2/ERK/PLD during sorbitol action, but largely downstream of the PI 3-kinase/PDK-1 during insulin action. Since aPKCs are required during activation of the glucose transport system by both sorbitol and insulin, these differences in signalling networks that are used to

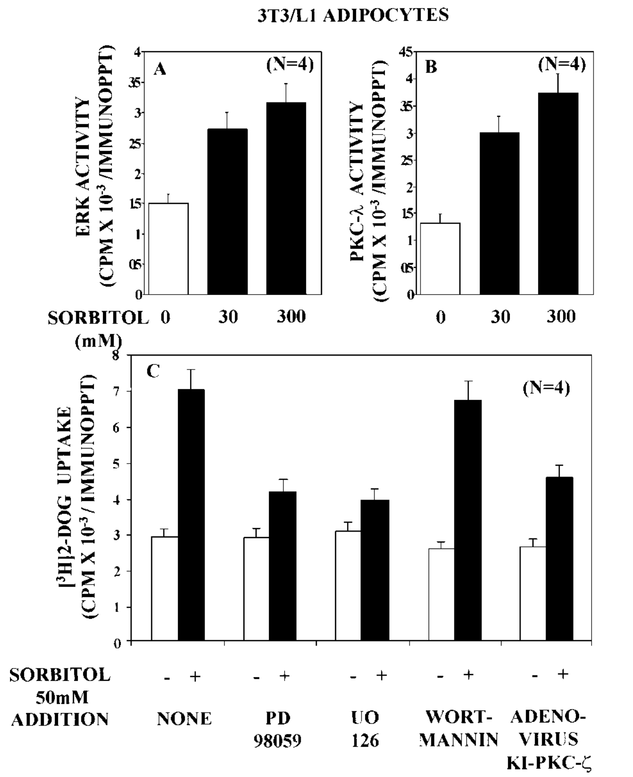

Figure 10 Effects of sorbitol and effects of inhibitors of MEK1 (25 μM PD98059 or 10 μM UO126), PI 3-kinase (100 nM wortmannin), and aPKCs (10 MOI adenovirus encoding KI-PKC-ζ) on sorbitol-induced increases in [^{3}H]2-DOG uptake in 3T3/L1 adipocytes

(**A**) ERK activity, (**B**) PKC-λ activity and (**C**) [^{3}H]2-DOG uptake. Fully differentiated adipocytes were first treated with adenovirus for 48 h to allow time for expression, or for 15 min with inhibitors, and then incubated with or without sorbitol for 15 min in enzyme activation studies, or for 30 min in glucose transport studies, following which, cell lysates were examined for immunoprecipitable ERK or aPKC activity, or [^{3}H]2-DOG uptake.

generate bioactive lipids, namely, PIP_3 and PA, which activate aPKCs, may account for the fact that activation of the ERK pathway results in increases in GLUT4 translocation/glucose transport during the action of sorbitol, but not insulin.

The activation of aPKCs by PI 3-kinase-dependent increases in acidic D3-PO_4 polyphosphoinositide phospholipids, in particular, PIP_3, and subsequent increases in the action of PDK-1, e.g. during insulin action [3–6,16–19], now seems clear. Moreover, this more conventional PIP_3-dependent mechanism is perhaps the only one generally well recognized to underlie the activation of aPKCs during agonist action. It was therefore interesting to find that sorbitol activated aPKCs through a novel mechanism that was dependent on the apparently sequential activation PYK2, the ERK pathway and PLD, and subsequent production of the acidic phospholipid, PA, which, as reported in [14,15] and at present confirmed, directly activates PKC-ζ.

It was in fact surprising to find that sorbitol-induced increases in PLD were dependent upon activation of the ERK pathway. On the other hand, ERK-dependent activation of PLD has also been seen in neutrophils during the action of the chemotactic tripeptide, *N*-formylmethionyl-leucyl-phenylalanine [24], which operates through a heterotrimeric G-protein-coupled receptor and activates PLD through several mechanisms [24–27]. Furthermore, RAS, which functions upstream of ERK, has also been reported to activate PLD [28], but it is not clear if ERK is required for this effect of RAS on PLD. Finally, we have recently reported that glucose, like sorbitol, activates the PYK2/ERK pathway [20]. Furthermore, like sorbitol, it activates PLD, PKC-ζ/λ and GLUT4 translocation in rat adipocytes and rat skeletal muscles [29]. Whether or not activation of aPKCs and glucose transport results from ERK-dependent or -independent PLD activation by other agonists/agents is at present uncertain.

The fact that aPKCs play a role in GLUT4 translocation/glucose transport, regardless of whether aPKCs are functioning downstream of PYK2/ERK/PLD or PI 3-kinase/PDK-1, is noteworthy. This increases the possibility that aPKCs may serve as common terminal activators of GLUT4 translocation for a variety of agonists that operate through different initial signalling mechanisms that generate specific bioactive lipids. Further work is needed to examine this possibility and determine whether other related kinases can substitute for aPKCs during GLUT4 translocation.

In summary, our results suggest that sorbitol activates the ERK pathway through PYK2, and this activation of PYK2/SOS/RAS/RAF/MEK1/ERK pathway results in the activation of PLD, which, via increases in PA, activates aPKCs and GLUT4 translocation/glucose transport in rat adipocytes, 3T3/L1adipocytes and L6 myotubes. Further studies are needed to determine how the ERK pathway activates PLD.

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