Insulin stimulation of K⁺ uptake in 3T3-L1 fibroblasts involves phosphatidylinositol 3-kinase and protein kinase C-zeta

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Summary Despite the important physiological role of insulin in the regulation of ionic homeostasis, primarily mediated by the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter, the intracellular signalling molecules mediating this effect of insulin have not been elucidated. Treatment of 3T3-L1 fibroblasts with insulin increased total ⁸⁶Rb⁺ (K⁺) uptake from 0.8 ± 0.04 to 1.02 ± 0.05 nmol · mg⁻¹ · protein⁻¹ · min⁻¹ ($p < 0.005$). These changes in K⁺ flux, though small, can alter the membrane potential. Uptake occurred through both the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter and both were stimulated by insulin. Interestingly, when bumetanide was used to inhibit the Na⁺/K⁺/2Cl⁻ cotransporter prior to insulin action, no increase in ⁸⁶Rb⁺ uptake via the Na⁺/K⁺-ATPase was observed. The structurally distinct phosphatidylinositol 3-kinase inhibitors wortmannin (50–200 nmol/l) and LY294002 (50 μmol/l) attenuated both total insulin-stimulated ⁸⁶Rb⁺ uptake as well as uptake via the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter. Nei-

ther the inhibitor of p70 S6 kinase activation, rapamycin (30 ng/ml) nor the mitogen activated protein kinase kinase inhibitor, PD098059 (50 μmol/l), had any effect on insulin's stimulation of K⁺ influx. A 10 μmol/l concentration of the protein kinase C (PKC) inhibitor bisindolylmaleimide attenuated insulin action but at 1 μmol/l it was ineffective, suggesting involvement of the atypical PKC-ζ isoform. We conclude that insulin-stimulated K⁺ uptake in 3T3-L1 fibroblasts appears to involve concerted regulation of both the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter and we show for the first time that this process is signalled via a pathway involving phosphatidylinositol 3-kinase and PKC-ζ. [Diabetologia (1998) 41: 1199–1204]

Keywords Na⁺/K⁺-ATPase, Na-pump, Na⁺/K⁺/2Cl⁻ cotransporter, insulin signalling, p70 S6 kinase, MAP kinase, PI 3-kinase, PKC-ζ.

The Na⁺/K⁺-ATPase represents the only route of Na⁺ efflux from most mammalian cells and is the major path for K⁺ uptake from the blood. It thus establishes and maintains the low intracellular Na⁺ and high intracellular K⁺ concentration observed under physio-

logical conditions [1]. The Na⁺/K⁺-ATPase is ubiquitously expressed and the functional enzyme is comprised of an α subunit, responsible for ion exchange and ATP hydrolysis and a glycosylated β subunit which is noncatalytic but confers stability to and modulates K⁺ transport by the α subunit [2]. To date, three isoforms of the α subunit and three isoforms of the β subunit have been identified and cloned in mammalian cells [3]. K⁺ also enters cells via the Na⁺/K⁺/2Cl⁻ cotransporter. This system catalyzes the concerted uptake of Na⁺, K⁺ and Cl⁻ in an electroneutral fashion [4], thus having a vital role in salt reabsorption or secretion and in maintaining cell volume. It is constituted by a single polypeptide with a high degree of glycosylation and two different types of Na⁺/K⁺

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Abbreviations: MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PI 3-kinase, phosphatidylinositol 3-kinase; BIM, bisindolylmaleimide; NKCC, Sodium Potassium Chloride Cotransporter

2Cl⁻ cotransporter have been described, termed secretory (NKCC1) and kidney specific absorptive (NKCC2) isoforms [4].

The activities of both the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter are regulated by various hormones [4, 5] and both activities are stimulated by insulin. Alteration of plasma membrane ion transport systems is among the most rapid responses of the cell to insulin and K⁺ uptake from plasma was one of the first reported actions of insulin [6]. The implications of insulin action on ion transport in diabetes are multiple. Increases in cell volume induced by insulin via alteration of ion transport have been shown in liver and it has been speculated that such changes are linked to metabolic regulation [7]. Hyperglycaemia inhibits Na⁺/K⁺-ATPase activity, and in streptozotocin diabetic rats an increase in [Na⁺]_i of 30% is commonly observed, followed by a decrease in pH_i with a consequent decrease in glycolysis, glucose oxidation and production of ATP [8]. Sodium retention also has a central role in the development of hypertension in diabetic subjects [9]. Other diabetic complications linked to altered ionic homeostasis include acromegaly, aldosteronism and obesity [10]. In contrast to the regulation of glucose uptake and metabolism, the signalling mechanisms used by insulin in controlling the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter are poorly characterized.

The purpose of this work, using 3T3-L1 fibroblasts, was to study the mechanisms whereby insulin regulates K⁺ uptake. We examined a) the contribution of the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter activities to the K⁺ flux and their inter-dependence and b) the role of constituents of insulin-stimulated signalling pathways such as phosphatidylinositol 3-kinase (PI 3-kinase), p70 S6 kinase, mitogen activated protein kinases (MAP) kinases and protein kinase C (PKC)- ζ in the activation of Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter.

Methods

⁸⁶Rb⁺ (K⁺) uptake assay in 3T3-L1 fibroblasts. Cells were grown in 24-well plates to assess ⁸⁶Rb⁺ uptake and were used 4 or 5 days postconfluence, as described previously [11]. Briefly, cells were preincubated in serum-free medium for 2 h, rinsed two times with N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES)-buffered saline solution (140 mmol/l NaCl, 2.4 mmol/l MgSO₄, 5 mmol/l KCl, 1 mmol/l CaCl₂ and 20 mmol/l HEPES, pH 7.4) then incubated at 37°C in 0.25 ml HEPES-buffered saline with or without inhibitors tested at the concentrations and for the times indicated in figure legends. This was followed by a 15 min incubation in the presence or absence of 100 nmol/l insulin, added directly to the HEPES-buffered saline. After this time ⁸⁶Rb⁺ was added in 50 μ l of HEPES-buffered saline to a final concentration of 5 μ Ci/ml. Uptake was allowed to proceed in the presence of inhibitor plus or minus insulin as indicated for 20 min, during which time uptake was linear, and terminated by washing three times

Fig. 1 Stimulation of ⁸⁶Rb⁺ uptake by insulin in 3T3-L1 fibroblasts. Uptake via the Na⁺/K⁺-ATPase (Pump) and Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) were calculated by subtracting ⁸⁶Rb⁺ uptake values obtained in the presence of ouabain (1.6 mol/l) and bumetanide (100 μ mol/l), respectively, from total uptake values obtained in their absence. Results are means $\pm$ SEM of at least three independent experiments. *** p < 0.005, ** p < 0.01 and * p < 0.05 relative to unstimulated cells

with 0.9% ice-cold saline. Cells were lysed with 1 ml of 0.05 mol NaOH and a 0.8 ml aliquot was counted using an LKB 1217 beta counter Fisher, Ont, Canada. Uptake via the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter were calculated, respectively, as the difference between total ⁸⁶Rb⁺ uptake and values obtained in the presence of 1.6 mmol/l ouabain and 100 μ mol/l bumetanide. Protein concentration of the lysate was determined using the Bradford method [12]. The pharmacological inhibitors were purchased from the following sources, wortmannin (Sigma, St. Louis, Mo., USA), LY294002 (Biomol, Plymouth Meeting, Pa., USA), BIM and rapamycin (Calbiochem, San Diego, Calif., USA) and PD098059 (a kind gift from Dr. A. Saltiel, Parke-Davis, Ann Arbor, Minn., USA).

Statistics. Statistical analysis was performed using the analysis of variance (Fisher, multiple comparisons).

Results

3T3-L1 fibroblasts express only α 1, β 1 and β 2 but not α 2 or α 3 subunits of the Na⁺/K⁺-ATPase (data not shown). The monoclonal antibody T4, specifically raised against the NKCC1 isoform, recognized a polypeptide of approximately 170 kDa corresponding to the Na⁺/K⁺/2Cl⁻ cotransporter in these cells (data not shown). Confirmation that both Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter polypeptides are expressed in 3T3-L1 fibroblasts prompted us to carry out functional studies analysing their activities and interdependence in response to insulin.

In Figure 1 we show that insulin increases total ⁸⁶Rb⁺ uptake in 3T3-L1 fibroblasts by almost 30%. Increases of comparable magnitude have been observed in kidney, muscle and fat and are of great physiological importance. The stimulation of K⁺

Fig. 2 Effect of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter inhibition on the stimulation by insulin of $^{86}\text{Rb}^+$ uptake via the $\text{Na}^+/\text{K}^+-\text{ATPase}$. Cells were treated with $100\ \mu\text{mol/l}$ bumetanide (Bu), in the presence or absence of insulin (Ins) for 15 min. Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was then determined. Uptake into untreated, unstimulated cells (Con) was assigned a value of 100%. Results are means $\pm$ SEM of three independent experiments. * $p < 0.05$ relative to the unstimulated value

(Rb^+) uptake into 3T3-L1 fibroblasts by insulin involved increases in both $\text{Na}^+/\text{K}^+-\text{ATPase}$ and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activity (assessed as ouabain-sensitive and bumetanide-sensitive uptake, respectively) (Fig. 1), confirming previous studies in human fibroblasts [13]. This suggests either a parallel stimu-

lation of the $\text{Na}^+/\text{K}^+-\text{ATPase}$ and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter or that the $\text{Na}^+/\text{K}^+-\text{ATPase}$ and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter lie in series. To address this question, fibroblasts were preincubated with bumetanide to inhibit $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activation upon stimulation with insulin, followed by assessment of ouabain-sensitive $^{86}\text{Rb}^+$ uptake. Figure 2 shows that the stimulation by insulin of the ouabain-sensitive component of $^{86}\text{Rb}^+$ uptake was inhibited by prior inhibition of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter. This result indicates that $\text{Na}^+/\text{K}^+-\text{ATPase}$ stimulation by insulin is dependent on $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activity. Conversely, when cells were preincubated with ouabain before insulin stimulation no effect was observed on subsequent $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activity (data not shown).

Two structurally distinct inhibitors of PI 3-kinases, wortmannin [14] and LY294002 [15], have been used extensively to define the role of this enzymatic activity in a variety of cellular functions such as glucose and amino acid transport [16]. Wortmannin, at a range of concentrations from 50–200 nmol/l inhibited insulin-stimulated total $^{86}\text{Rb}^+$ uptake (by 78 % at 50 nmol/l 86 % at 100 nmol/l and fully at 200 nmol/l (Fig. 3a). LY294002 (50 nmol/l) inhibited insulin stimulation of total $^{86}\text{Rb}^+$ uptake by 83 % (Fig. 3a). When the component of $^{86}\text{Rb}^+$ uptake mediated by the $\text{Na}^+/\text{K}^+-\text{ATPase}$ was calculated, both wortmannin and LY294002 were seen to exert profound inhibitory effects on insulin-stimulated activity (Fig. 3b). Insulin-stimulated $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activity was also suppressed by wortmannin and LY294002, although to a somewhat lesser degree in the latter case (Fig. 3c). Higher concentrations of LY294002 were not used because of potential non-specific effects and as this concentration has been shown to be sufficient for specific PI 3-kinase inhibition. Neither wortmannin nor LY294002 appreciably altered the basal rate of total, ouabain-sensitive, or bumetanide-sensitive $^{86}\text{Rb}^+$ uptake components (data not shown).

The p70 S6 kinase signalling pathway mediates a diverse array of cellular responses [17]. This kinase is regulated by phosphorylation and it has been suggested that it lies downstream of PI 3-kinase in signal-

Fig. 3A, B, C Effect of wortmannin and LY294002 on the stimulation by insulin of $^{86}\text{Rb}^+$ uptake in 3T3-L1 fibroblasts. Insulin-stimulated $^{86}\text{Rb}^+$ uptake was assessed as total (A), ouabain-sensitive (B) and bumetanide-sensitive (C) as described in "Materials and methods". In each case the insulin-stimulated increase in uptake was assigned a value of 100 %. Inhibition of this value towards basal (0 %) was calculated after a 20 min preincubation of cells with wortmannin (WM) at concentrations of 0.05 $\mu\text{mol/l}$, 0.1 $\mu\text{mol/l}$ and 0.2 $\mu\text{mol/l}$ or after preincubation with LY294002 (LY) at 50 $\mu\text{mol/l}$ for 20 min. Results are means $\pm$ SEM of at least three independent experiments. *** $p < 0.005$, and * $p < 0.05$ relative to the insulin-stimulated value

Table 1 Effect of rapamycin and PD098059 on the stimulation by insulin of ⁸⁶Rb⁺ uptake in 3T3-L1 fibroblasts. Insulin-stimulated total uptake, uptake via the Na⁺/K⁺-ATPase and via the Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) were assessed as described in “Materials and methods”. For each determination the insulin-stimulated uptake was assigned a value of 100 %. The effect of insulin was also calculated after preincubation of cells with 30 ng/ml rapamycin for 20 min and after preincubation with 50 μmol/l PD098059 for 30 min. Results are means ± SEM of at least three independent experiments

⁸⁶ Rb ⁺ uptake	Insulin-stimulated	Insulin + rapamycin	Insulin + PD098059
Total	100 %	104 ± 5	86 ± 8
Via Na ⁺ /K ⁺ -ATPase	100 %	108 ± 9	95 ± 8
Via NKCC	100 %	87 ± 5	131 ± 24

ling pathways triggered by insulin and platelet derived growth factor [18]. Rapamycin is used to selectively inhibit activation of p70 S6 kinase by growth factors [19]. Despite its extensive use the effect of rapamycin on p70 S6 kinase activity has not been characterized in 3T3-L1 fibroblasts. We therefore confirmed the effectiveness of rapamycin in these cells by measuring its ability to inhibit insulin-stimulated p70 S6 kinase activity. In two separate experiments rapamycin did inhibit p70 S6 kinase activation by insulin in these cells [average values: control = 1 (arbitrary unit), insulin = 2.8 (*p* < 0.05), insulin + rapamycin = 0.9]. Rapamycin had no effect on insulin-stimulated total ⁸⁶Rb⁺ uptake, nor on uptake via the Na⁺/

K⁺-ATPase or the Na⁺/K⁺/2Cl⁻ cotransporter (Table 1). In addition, rapamycin did not have any effect on basal total, ouabain-sensitive, or bumetanide-sensitive ⁸⁶Rb⁺ uptake levels (data not shown).

Modulation of the MAP kinase cascade by insulin has been extensively reported and it has been suggested that this can also occur via PI 3-kinase [20]. We tested the possible involvement of this pathway in the stimulation of the Na-pump and Na⁺/K⁺/2Cl⁻ cotransporter by insulin. The mitogen-activated protein kinase kinase (MEK) inhibitor PD098059 (Table 1) had no effect on insulin-stimulated total or ouabain-sensitive ⁸⁶Rb⁺ uptake. Although a slightly increased level of insulin-stimulated bumetanide-sensitive activity was apparent in the presence of PD098059, this difference was not statistically significant. PD098059 did not have any effect on basal total, ouabain-sensitive, or bumetanide-sensitive ⁸⁶Rb⁺ uptake levels (data not shown). The inability of PD098059 to inhibit insulin action was substantiated by studies showing the effect of this inhibitor on insulin-stimulated phosphorylation of the MEK substrates extracellular-regulated kinase (ERK)1 and ERK2 in these cells. We confirmed that PD098059 was capable of reducing insulin-induced phosphorylation of ERK1 and ERK2 (data not shown).

Emerging evidence shows that PKC, in particular the PKC-ζ isoform, is a component of an insulin-stimulated signalling cascade [21]. Regulation of the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ cotransporter by the PKC family is a well characterized phenomenon [5, 22]. We confirmed that 3T3-L1 fibroblasts express PKC-α, PKC-β, PKC-ε, and PKC-ζ (data not shown). Bisindolymaleimide (BIM) has been reported to specifically inhibit most PKC isoforms at a concentration of 10 μmol/l or above whereas when used at 1 μmol/l, PKC-ζ is not susceptible to inhibition but other isoforms are [23]. We found that a concentration of 10 μmol/l BIM was necessary to inhibit insulin-stimulated total ⁸⁶Rb⁺ uptake, whereas the insulin response was not affected by 1 μmol/l BIM (Fig. 4a). This effect on total ⁸⁶Rb⁺ uptake was mirrored in studying uptake specifically via the Na⁺/K⁺-ATPase

Fig. 4A, B, C Effect of bisindolymaleimide on the stimulation by insulin of ⁸⁶Rb⁺ uptake in 3T3-L1 fibroblasts. Insulin-stimulated ⁸⁶Rb⁺ uptake was assessed as total (A), ouabain-sensitive (B) and bumetanide-sensitive (C) as described in “Materials and methods”. In each case the insulin-stimulated increase in uptake was assigned a value of 100 %. Inhibition of this value towards basal (0 %) was calculated after preincubation of cells with bisindolymaleimide (BIM) at concentrations of 1 μmol/l and 10 μmol/l for 20 min. Results are means ± SEM of at least three independent experiments. ****p* < 0.005, ***p* < 0.01 and **p* < 0.05 relative to the insulin-stimulated value

or $Na^+/K^+/2Cl^-$ cotransporter (Fig. 4). Bisindolylmaleimide had no effect on basal rates of total, ouabain-sensitive, or bumetanide-sensitive $^{86}Rb^+$ uptake components (data not shown).

Discussion

As the experimental manipulations used here were not amenable to using an animal model, we chose to use 3T3-L1 fibroblasts as a model system for this study as it is a commonly used insulin-responsive cell line. Because of the existence of several α and β subunit isoforms and their differential expression, regulation of the Na^+/K^+ -ATPase is tissue specific. It was therefore important to characterize the expression of Na^+/K^+ -ATPase isoforms and $Na^+/K^+/2Cl^-$ cotransporter in 3T3-L1 fibroblasts. These cells were found to express the $\alpha 1$ but not $\alpha 2$ or $\alpha 3$ subunits, confirming previous observations [11, 24]. The present study also detected $\beta 1$ and $\beta 2$ subunits (data not shown). The functional pump units in these cells could therefore be $\alpha 1:\beta 1$ and $\alpha 1:\beta 2$, since all α isoforms appear to be capable of interacting with all β subunit isoforms [25]. Thus it is important to keep in mind that when analysing mechanisms of insulin action in this study we are looking at regulation of the $\alpha 1$ isoform. This is also the α isoform of kidney; in contrast, in skeletal muscle $\alpha 1$ and $\alpha 2$ coexist and $\alpha 2$ translocates to the cell surface in response to insulin [3].

The Na^+/K^+ -ATPase activity is low under resting conditions and can be regulated by several hormones, notably catecholamines and insulin [5]. Even though the stimulation elicited by hormones is only approximately 5% of the increase in Na^+/K^+ -ATPase activity caused by maximal electrical stimulation of muscle [26], hormonal regulation remains a key mechanism for the control of Na^+ and K^+ gradients. Stimulation of the Na^+/K^+ -ATPase by insulin has been documented in rat adipocytes, skeletal muscle, kidney, liver and in cells in culture such as hepatocytes and 3T3-L1 adipocytes where responses of comparable magnitude to those reported here were observed [3]. In contrast, the regulation of $Na^+/K^+/2Cl^-$ cotransporter activity has been studied mostly in response to catecholamines, although it has been shown that insulin increases K^+ uptake via a bumetanide-sensitive pathway in liver [27], 3T3-L1 adipocytes [11] and human fibroblasts in culture treated with micromolar concentrations of the hormone [13]. Figure 1 shows that insulin stimulates $^{86}Rb^+$ uptake into 3T3-L1 fibroblasts and that this increase comprises both ouabain-sensitive and bumetanide-sensitive components. This suggests either a parallel stimulation of the Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ cotransporter or that the Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ cotransporter lie in series. Since resting $[Na^+]_i$ levels are below threshold for maximal Na^+/K^+ -ATPase activity, a rise

in $[Na^+]_i$ can increase Na^+/K^+ -ATPase activity. There is a precedent for this means of activation since stimulation of the Na^+/K^+ -ATPase subsequent to increased $[Na^+]_i$ (via Na^+/H^+ exchange) is apparent in rat hepatocytes [28] and BC3H-1 myocytes [29] and also (via a Na^+ channel) in 3T3-F442 cells [30]. The results presented in Figure 2 suggest that an increased intracellular Na^+ concentration, in this case as a consequence of $Na^+/K^+/2Cl^-$ cotransporter stimulation, could perhaps underlie the stimulation of the Na^+/K^+ -pump by insulin in 3T3-L1 fibroblasts. The rise in intracellular Na^+ could be envisaged to stimulate K^+ (Rb^+) uptake by mass action or via an allosteric effect or both. Concerted regulation of the Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ cotransporter would be of particular physiological importance in kidney tubule cells where the $Na^+/K^+/2Cl^-$ cotransporter is predominantly expressed on the apical surface and the Na^+/K^+ -ATPase on the basolateral surface resulting in efficient reabsorption of Na^+ from the lumen to the blood [31]. Our findings might also help explain the widespread observation that the actions of insulin are greatly impaired when extracellular Na^+ is limiting [7].

Another objective of this study was to investigate the intracellular signalling pathway used by insulin in mediating stimulation of K^+ uptake. PI 3-kinase has been established as a key molecule in regulating insulin-stimulated processes such as glucose transport [32] and we show that PI 3-kinase inhibition prevents the insulin-stimulated increase in K^+ uptake in 3T3-L1 fibroblasts. It has been suggested that PKC- ζ lies downstream of PI 3-kinase as it is stimulated by the products of PI 3-kinase in vitro [33], both PIP3 and PI(3,4)P2 specifically stimulating PKC- ζ yet having negligible effects on most other PKC isoforms [34]. Most recently, wortmannin and LY294002 were shown to abrogate lipopolysaccharide induced translocation and activation of PKC- ζ in human monocytes [35] and wortmannin inhibited nuclear translocation of PKC- ζ during ischaemia in rat cardiac tissue [36]. For many years now PKC has been proposed as both a mediator and inhibitor of insulin action, the contrasting results being attributed to differential expression of individual isoforms in different tissues [37]. A wealth of evidence now suggests PKC- ζ could be the isoform used by insulin in many cells as part of its intracellular signalling pathway. This isoform is required for stimulation of protein synthesis by insulin [38] and the hormone leads to a rapid translocation of both PKC- ζ and PI 3-kinase in fibroblasts [39]. Our results using the PKC inhibitor bisindolylmaleimide (BIM) imply a role for PKC- ζ in regulation of K^+ uptake by insulin in 3T3-L1 fibroblasts. A difference in IC_{50} for PKC- ζ compared with other isoforms allows this isoform to be discriminated by concentrations of 1 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$ BIM [23]. For stimulation of K^+ uptake in these cells we found that 1 $\mu\text{mol/l}$ BIM had no effect on insulin action but 10 $\mu\text{mol/l}$ was

inhibitory. These observations are strengthened by the recent observation that PKC- ζ can be stimulated by insulin in these cells [21] and that the PI 3-kinase inhibitor wortmannin prevented insulin-stimulated activation of PKC- ζ [38, 40].

In conclusion, our results highlight the novel observation that PI 3-kinase, and most likely PKC- ζ , are key intracellular regulatory molecules in the stimulation of K⁺ uptake into 3T3-L1 fibroblasts by insulin. This effect is mediated via Na⁺/K⁺/2Cl⁻ cotransporter stimulation with subsequent stimulation of the Na-pump. Insulin does not employ p70 S6 kinase nor the MAP kinase cascade to elicit this response. These observations could have implications in diabetes-related disorders involving defects in ionic homeostasis such as cardiovascular disease, hypertension, obesity, nephropathy and neuropathy.

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