Insulin increases plasma membrane content and reduces phosphorylation of Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit in HEK-293 cells

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Insulin increases plasma membrane content and reduces phosphorylation of Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit in HEK-293 cells. \textit{Am J Physiol Cell Physiol} 281: C1797--C1803, 2001.—Insulin stimulates K\textsuperscript{+} uptake and Na\textsuperscript{+} efflux via the Na\textsuperscript{+}-K\textsuperscript{+} pump in kidney, skeletal muscle, and brain. The mechanism of insulin action in these tissues differs, in part, because of differences in the isoform complement of the catalytic \(\alpha\)-subunit of the Na\textsuperscript{+}-K\textsuperscript{+} pump. To analyze specifically the effect of insulin on the \(\alpha_1\)-isoform of the pump, we have studied human embryonic kidney (HEK)-293 cells stably transfected with the rat Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-isoform tagged on its first exofacial loop with a hemagglutinin (HA) epitope. The plasma membrane content of \(\alpha_1\)-subunits was quantitated by binding a specific HA antibody to intact cells. Insulin rapidly increased the number of \(\alpha_1\)-subunits at the cell surface. This gain was sensitive to the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin and to the protein kinase C (PKC) inhibitor bisindolylmaleimide. Furthermore, the insulin-stimulated gain in surface \(\alpha\)-subunits correlated with an increase in the binding of an antibody that recognizes only the nonphosphorylated form of \(\alpha_1\) (at serine-18). These results suggest that insulin regulates the Na\textsuperscript{+}-K\textsuperscript{+} pump in HEK-293 cells, at least in part, by decreasing serine phosphorylation and increasing plasma membrane content of \(\alpha_1\)-subunits via a signaling pathway involving PI 3-kinase and PKC.

ouabain; phosphatidylinositol 3-kinase; protein kinase C; hemagglutinin

The sodium-potassium pump (Na\textsuperscript{+}-K\textsuperscript{+}-ATPase) expels three Na\textsuperscript{+} ions from the cytosol in exchange for two extracellular K\textsuperscript{+} ions (28). This electrogenic process uses ATP and represents the only means of Na\textsuperscript{+} extrusion from cells. Changes in pump activity directly impact on processes dependent on Na\textsuperscript{+} and K\textsuperscript{+} gradients (e.g., the resting membrane potential, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, and coupled transport of glucose, vitamins, and amino acids).

The mature functional Na\textsuperscript{+}-K\textsuperscript{+} pump requires association of its \(\alpha\)- and \(\beta\)-subunits (28). The 110-kDa \(\alpha\)-subunit is the catalytic component, whereas the highly glycosylated \(\beta\)-subunit (apparent molecular weight on SDS-PAGE of \(\sim\)55 kDa) is involved in maturation and assembly of functional pump dimers in the plasma membrane (32), in allowing K\textsuperscript{+} occlusion (29), and in modulation of Na\textsuperscript{+} and K\textsuperscript{+} affinity of the enzyme (15, 16). Four isoforms of \(\alpha\)-subunits and three of \(\beta\)-subunits exist (6). In kidney, the pump also comprises a \(\gamma\)-subunit, a small (8–14 kDa) hydrophobic peptide that may regulate affinity of the \(\alpha\)-subunit for ATP, Na\textsuperscript{+}, and K\textsuperscript{+} (1, 2, 39). The \(\alpha\)- and \(\beta\)-isoforms exhibit a tissue-specific distribution, and their heterogeneity contributes to the adaptability of the Na\textsuperscript{+}-K\textsuperscript{+} pump to respond to hormones in a tissue-specific fashion (6, 19).

A diverse range of hormones can regulate Na\textsuperscript{+}-K\textsuperscript{+} pump activity acutely or chronically (5, 19). Short-term regulation may be achieved by several mechanisms including changes in ion affinity and regulation of availability of pump subunits at the cell surface through exocytosis or endocytosis. It has been proposed that changes in phosphorylation of the \(\alpha\)-subunit may contribute to regulation of Na\textsuperscript{+}-K\textsuperscript{+} pump localization by dopamine in kidney proximal tubule (11, 12). On the other hand, several studies have established negative correlations between the phosphorylation of a serine residue in the NH\textsubscript{2} terminus on the \(\alpha_1\)-subunit and its level of catalytic activity (4, 24, 33).

Regulation of the Na\textsuperscript{+}-K\textsuperscript{+} pump by insulin also occurs through diverse, tissue-specific mechanisms (36). In skeletal muscle, insulin causes translocation of the \(\alpha_2\)-isoform to the cell surface (25, 31). In contrast, no change in \(\alpha_1\)- or \(\alpha_2\)-subunit exposure was noted in 3T3-L1 fibroblasts or adipocytes, respectively, where instead the pump was activated secondarily to a rise in intracellular Na\textsuperscript{+} concentration (30, 35, 37). In the proximal convoluted tubule of the kidney, insulin activates the Na\textsuperscript{+}-K\textsuperscript{+} pump by elevating the affinity for Na\textsuperscript{+} of the \(\alpha_1\)-subunit (21). Recently, tyrosine phosphorylation of the \(\alpha_1\)-subunit was shown to correlate positively with increased pump activity in response to insulin in kidney proximal tubule (20). Although the
insulin receptor is itself a tyrosine kinase, once activated it engages signaling cascades involving the lipid kinase phosphatidylinositol (PI) 3-kinase and the serine/threonine kinases Akt and atypical protein kinase C (PKC), as well as serine/threonine and tyrosine phosphatases (38).

Clearly, there is a need to identify the mechanism of regulation of each Na⁺-K⁺ pump isoform by specific stimuli. The objective of this study was to explore whether insulin can cause changes in the surface exposure of the Na⁺-K⁺ pump α₁-subunit. For this purpose, we used human embryonic kidney (HEK)-293 cells stably transfected with an exofacially epitope-tagged rat Na⁺-K⁺ pump α₁-isooform in combination with an intact cell assay to examine its presence at the plasma membrane. Furthermore, we measured changes in α₁-subunit phosphorylation and examined the possible signaling pathways involved in the response to insulin.

MATERIALS AND METHODS

Materials. All cell culture solutions and supplements were obtained from GIBCO BRL (Burlington, ON, Canada). HEK cells stably overexpressing the hemagglutinin (HA)-tagged rat Na⁺-K⁺ pump α₁-isooform protein [HA (2x) 119 I] were generated as previously described (8). Human insulin (Humulin) was obtained from Eli Lilly Canada (Toronto, ON, Canada). Protein A- and protein G-Sepharose were from Pharmacia (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham (Oakville, ON, Canada). The Na⁺-K⁺ pump α₁-subunit antibody McK1 (22) was a generous gift from Dr. K. Sweadner (Massachusetts General Hospital, Boston, MA). Anti-HA antibody (12CA5) was purchased from Roche Diagnostics (Quebec, Canada). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and donkey anti-mouse antisera were from Jackson ImmunoResearch (Baltimore Pike, PA). o-Phenylenediamine dihydrochloride (OPD reagent) and wortmannin were from Sigma (St. Louis, MO), and bis-dimethylmaleimide (BIM) was from Calbiochem (San Diego, CA). All electrophoresis and immunoblotting reagents were purchased from Bio-Rad (Mississauga, ON, Canada). All other reagents were of the highest analytical grade.

Cell culture. HEK-293 cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) in an atmosphere of 5% CO₂ at 37°C. Transfection of the HA (2x) 119 I mutant into HEK-293 cells conferred ouabain resistance; hence, cells were grown in the presence of 0.5 μM ouabain. For α₁-subunit translocation assays, cells were seeded in 12-well plates (2.5-cm-diameter well). Cells were maintained under the same conditions in six-well plates for preparation of whole cell lysates and subsequent immunoprecipitation.

Immunodetection of Na⁺-K⁺ pump α₁-subunit translocation to the plasma membrane. Stably transfected HEK cells were seeded on 12-well plates at a high density and used 1–2 days postconfluence. Before each experiment, cells were depleted of serum for 2 h. Cells were then incubated with inhibitors and/or insulin for the desired time and rinsed quickly in PBS, and then 300 μl of 3% paraformaldehyde were added per well for 3 min at 4°C. In initial experiments, similar results were obtained when paraformaldehyde was added before or after incubation with antibody, suggesting that permeabilization of cells with paraformaldehyde was not significant. Paraformaldehyde was aspirated, and 500 μl of 1% glycine in PBS were added for 10 min. Each well was then rinsed once with 1 ml PBS before 300 μl of 5% goat serum plus 3% BSA in PBS were added for 30 min. After this period, 300 μl of anti-HA monoclonal antibody (12CA5) were added (1,500,000 dilution in 5% goat serum + 3% BSA) for 30 min, followed by extensive washing (3 times with 1 ml of PBS per well). Peroxidase (300 μl)-conjugated donkey anti-mouse IgG (1,500,000 dilution in 5% goat serum + 3% BSA) was added for 30 min, again followed by extensive washing. Finally, each well was incubated with 1 ml of OPD reagent for 12 min, at which time 0.25 ml of 3 M HCl was added, and the solution was transferred to a cuvette for absorbance reading at 492 nm. Background values were calculated by using the same procedure but omitting primary antibody, and basal values were calculated by subtracting this background value from those obtained with transfected but untreated cells. To estimate the percentage of total cellular HA-tagged α₁-subunits expressed at the cell surface, we employed a laser method with the addition of a permeabilization step, using 0.1% Triton X-100, after cells were fixed by paraformaldehyde. We found that approximately one-third of these subunits were inserted in the plasma membrane under resting conditions.

Assay of PI 3-kinase activity associated with anti- phosphotyrosine. PI 3-kinase activity was measured on phosphotyrosine immunoprecipitates as described previously (41). Briefly, the ability of PI 3-kinase associated with phosphotyrosine to convert phosphatidylinositol to phosphatidylinositol-3-phosphate (PI3P) was detected by separation of these lipids by thin-layer chromatography (TLC). Detection and quantitation of [32P]PI3P on the TLC plates were done using a Molecular Dynamics PhosphorImager system (Sunnyvale, CA).

Assay of atypical PKC activity. Immunoprecipitation of atypical PKC isoforms was performed with antibody PKC-ζ (C-20; Santa Cruz, CA), which recognizes both PKC-ζ and PKC-λ. Kinase assays were performed by lysing cells in buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM glycerol (vol/vol), 1% Triton X-100 (vol/vol), 30 mM Na₃PO₄, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 mM Na₃VO₄, 1 mM dithiothreitol (DTT), and 100 mM okadaic acid. Antibody (2 μg per condition) precooled to protein A-protein G-Sepharose [20 μl (100 mg/ml) each per condition] beads was added to 200 μg of total protein from cell lysates. Antibody-coupled beads were washed twice with ice-cold PBS and once with ice-cold lysis buffer before use. PKC-ζ/λ was immunoprecipitated by incubation with the antibody-bead complex for 2–3 h under constant rotation (4°C). Immunocomplexes were isolated and washed four times with 1 ml of wash buffer (25 mM HEPES, pH 7.8, 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 0.1% BSA, 1 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM microcystin, and 100 mM okadaic acid) and twice with 1 ml of kinase buffer (50 mM Tris·HCl, pH 7.5, 10 mM MgCl₂, 10 mM okadaic acid, and 1 mM DTT). The complexes were then incubated under constant agitation for 30 min at 30°C with 30 μl of reaction mixture (kinase buffer containing 5 μM ATP, 2 μg [γ³²P]ATP, and 5 μg of myelin basic protein).

After the reaction, 30 μl of the supernatant were transferred onto Whatman p81 filter paper and washed four times for 10 min with 3 ml of 175 mM phosphoric acid and once with distilled water for 5 min. Filters were air-dried and then subjected to liquid scintillation counting.
were lysed in 1 ml of lysis buffer [50 mM HEPES, pH 7.6, 150 mM NaCl, 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 30 mM Na3P2O7, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM NaN3VO4, 1 mM DTTC, and 100 nM okadaic acid] and passed five times through a 25-gauge syringe needle. To each lysate, we added 2 μg of HA antibody and then incubated overnight with constant rotation, followed by 25 μl each of protein A- and protein G-Sepharose (10% wt/vol) for 1 h. Immunoprecipitates were then washed three times with PBS containing 0.1% Nonidet P-40 and 100 μM NaN3VO4, solubilized in 30 μl of 2× Laemmli sample buffer, and incubated at 40°C for 20 min before separation by 10% SDS-PAGE. Monoclonal α1-subunit antibodies 6H (1:1,000 dilution) or McK1 [1:4,000 dilution, which recognizes only rat Na+–K+ pump α1 protein with nonphosphorylated serine-18 (9, 23)] were used for immunoblotting, followed by sheep anti-mouse immunoglobulin conjugated to HRP (1:5,000 dilution), and protein was visualized by the ECL method.

Statistical analysis. Statistical analysis was performed by using either unpaired Student’s t-test or analysis of variance (Fischer, multiple comparisons) where appropriate, as indicated.

RESULTS

The HEK-293 cells transfected with HA-tagged Na+–K+ pump α1-isoform were developed to localize cytoplasmic and extracellular domains of the Na+–K+ pump (8). In that study, analysis of cells expressing the HA (2x) 119 I rat Na+–K+ pump α1-subunit mutant constructs showed reactivity with HA antibody in both permeabilized and intact cells, indicating an extracellular location of the HA epitope. These cells are thus amenable to analysis of exposure of Na+–K+ pump α1-isoform at the cell surface by detecting HA epitope availability in intact cells. We have developed an assay where cell monolayers are fixed before incubation with HA antibody and then exposed to an appropriate HRP-coupled secondary antibody. Peroxidase activity is detected in a colorimetric assay using the OPD reagent (42). Figure 1A shows that insulin elicited a rapid, yet transient increase in the amount of HA-tagged α1-subunits in the plasma membrane. The effect of insulin was apparent within minutes, peaked at around 10 min to achieve an ~1.4-fold stimulation above basal levels, and returned to basal levels after 20 min. The magnitude of this response is typical of increases in ion transport via the Na+–K+ pump seen in response to insulin and other hormones (35, 37). In keeping with most studies in the literature in which insulin-responsive cells in culture such as 3T3-L1 adipocytes and L6 myotubes were used, most other experiments performed here were done using 100 nM insulin. However, treatment with increasing concentrations of insulin for 10 min showed that 0.1 nM insulin elevated α1-subunit surface exposure significantly and that 1 nM caused maximal stimulation (Fig. 1B). This high sensitivity of HEK-293 cells suggests that they are indeed a good in vitro model of insulin action and that they retain the high affinity for the hormone displayed by mature tissues.

The signaling pathways engaged by insulin to stimulate the increase in α1-subunits to the plasma membrane in these cells was investigated next. Previous studies by us and others (13, 14, 34, 37) have suggested the participation of PI 3-kinase in mediating the stimulation of Na+–K+ pump activity in fibroblasts and epithelial cells. Therefore, we examined the effect of the PI 3-kinase inhibitor wortmannin on the gain in α1-subunits at the plasma membrane of HEK-293 cells. Figure 2A shows that preincubation of cells with 100 nM wortmannin prevented the insulin-induced gain in α1-subunits at the cell surface without significantly affecting basal levels. Figure 2B demonstrates that insulin stimulated phosphotyrosine-associated PI 3-kinase activity. This response occurred with a time course similar to that observed for stimulation of α1 translocation.

Atypical PKC isoforms are activated by the lipid products of PI 3-kinase (40). We also have suggested previously that atypical PKC contributes to the stimulation of the Na+–K+ pump by insulin in fibroblasts (37) and insulin-like growth factor (IGF)-I in smooth muscle cells (27) on the basis of the sensitivity of the response to micromolar concentrations of the PKC inhibitor BIM. Here we show that insulin stimulates PKC-ζ activity in HEK-293 cells as measured by the...
ability of immunoprecipitates to phosphorylate myelin basic protein; this activity was inhibited by 10 μM BIM (Fig. 3B). At this concentration BIM also caused inhibition of insulin-stimulated α1-subunit content in the plasma membrane (Fig. 3A). A lower concentration of BIM (1 μM), which spares atypical PKCs but inhibits conventional and novel PKCs, partially inhibited the insulin-stimulated increase in cell surface α1-subunits [insulin (10 min): 100 ± 3%; insulin + 1 μM BIM: 66 ± 5%; and insulin + 10 μM BIM: 12 ± 9%]. Collectively, these results suggest that PKC may participate in relaying the insulin signal to the Na+-K+ pump in HEK cells.

To explore whether the phosphorylation status of the α1-subunit is affected by insulin, we made use of an antibody (McK1) that preferentially recognizes the nonphosphorylated form of this subunit (23). The use of McK1 as a tool to investigate phosphorylation of the α1-subunit has been validated several times (9, 26). The antibody specifically recognizes the nonphosphorylated form of the enzyme on serine-18, a residue that is a target for phosphorylation by PKC (23, 26). This sequence is unique to the rat α1-isoform (22), and the HA-tagged α1-subunit expressed in HEK cells used here is of rat origin; therefore, it is possible to detect changes in its α1-subunit phosphorylation using McK1 antibody. Serine-18 phosphorylation of HA-tagged α1-subunit of HEK-293 cells was determined by immunoprecipitating the protein with anti-HA antibody, followed by immunoblotting with McK1. Insulin caused a 30% increase in McK1 immunoreactivity (Fig. 4). The presence of equivalent total amounts of α1-subunit protein content in all samples was confirmed with the use of a monoclonal antibody (6H) that does not discriminate between phosphorylated and nonphosphorylated protein (Fig. 4, inset). The effect of insulin was attenuated by both wortmannin and BIM (Fig. 4). In experiments similar to that illustrated in Fig. 4, we were unable to detect any insulin-dependent tyrosine phosphorylation of the α1-subunit by immunoblotting HA immunoprecipitates with anti-phosphotyrosine antibody (results not shown).

DISCUSSION

The studies described in the introduction and the results presented in this study document a very versatile regulation of the Na+-K+ pump. It is clear that changes in cellular localization of specific isoforms of the α-subunit, along with changes in its phosphorylation status, contribute to the ultimate ability of the cells to regulate Na+ efflux and K+ influx. Until now, other studies had made use of subcellular fractionation and morphological analysis by immunocytochemistry to analyze changes in cellular localization. Neither of these techniques resolve the issue of whether or how many of the pump subunits are truly inserted into the plasma membrane or are in docked but not fused subplasmalemmal vesicles. Direct approaches to detect pump exposure at the exofacial face of the membrane...
Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition in response to dopamine in proximal tubule cells (13). It also has been shown that activation of PKC-\(\zeta\) by dopamine resulted in inhibition of renal Na\textsuperscript{+}-K\textsuperscript{+} pump (17). In contrast, PKC-\(\beta\) activation appears to be responsible for increased renal pump phosphorylation and activity in response to phorbol 12-myristate 13-acetate (17, 18), suggesting that the role of PKC in regulating the Na\textsuperscript{+}-K\textsuperscript{+} pump is complex and likely isoform and tissue specific. Here we demonstrated that insulin can stimulate phosphotyrosine-associated PI 3-kinase activity and atypical PKC activity in HEK-293 cells. We then showed that inhibitors of these enzymes, i.e., wortmannin (100 nM) and BIM (10 \(\mu\)M), prevented insulin-induced increases in HA-tagged \(\alpha_1\)-subunit exposure at the cell surface. These results suggest that the insulin-signaling pathway regulating increased Na\textsuperscript{+}-K\textsuperscript{+} pump function may involve PI 3-kinase and PKC. Indeed, the temporal activation of these lipid and serine kinases in these experiments and the transient nature of HA-tagged \(\alpha_1\)-subunit translocalization also provides correlative evidence for a close association of these phenomena.

Wortmannin and BIM also reduced the ability of insulin to reduce serine phosphorylation of \(\alpha_1\)-subunits. The fact that insulin stimulated PKC activity yet ultimately caused a reduction in serine phosphorylation of the \(\alpha_1\)-subunit suggests that a phosphatase may lie downstream of PKC in this insulin-signaling pathway. Phosphatase activation as a distal event in kinase cascades is common, and indeed, insulin activates several serine phosphatases, including protein phosphatase-1 (PP1) (3). Notably, activation of PP1 in muscle cells was wortmannin sensitive (14, 34), but it is not known whether its activation requires PKC activity. We hypothesize that activation of PI 3-kinase and PKC by insulin result in increased phosphatase activity and dephosphorylation of the \(\alpha_1\)-subunit of the Na\textsuperscript{+}-K\textsuperscript{+} pump in HEK-293 cells. It previously has been shown that the phosphorylation status of the \(\alpha_1\)-isoform can be altered as a result of PKA or PKC activation (7, 22), and in some cases changes in phosphorylation of the pump have been linked to changes in \(\alpha_1\) localization. Notably, dopamine-induced phosphorylation of \(\alpha_1\) by PKC accompanies increased endocytosis of this subunit in proximal kidney tubules (10). The clathrin-dependent endocytosis of \(\alpha_1\) into endosomes required phosphorylation of \(\alpha_1\) at serine-18 (11). These studies suggest that phosphorylation constitutes a signal for Na\textsuperscript{+}-K\textsuperscript{+} pump endocytosis (12). In the present study, insulin appeared to reduce the phosphorylation of serine-18. This might explain the increased cell surface content of the \(\alpha_1\)-subunit if indeed phosphorylation of this residue is a cause of increased endocytosis of the protein. Consistent with this scenario, the magnitude of the insulin-dependent dephosphorylation of the \(\alpha_1\)-subunit (30%) closely correlated with the gain in surface exposure of this protein (40%).

In summary, the results of this study suggest that insulin leads to a gain in Na\textsuperscript{+}-K\textsuperscript{+} pump at the cell surface that may be linked to the reduction in phosphorylation of serine-18 on the \(\alpha_1\)-subunit. The mech-

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**Fig. 4. Effect of insulin on serine phosphorylation of the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit in HEK-293 cells on the basis of immunoreactivity to McK1 antibody.** Cells were pretreated with wortmannin (100 nM, 30 min) or BIM (10 \(\mu\)M, 20 min) and then treated with insulin (100 nM, 10 min). Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunits were immunoprecipitated using HA antibody as described in MATERIALS AND METHODS. Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting with McK1 (1:4,000), which recognizes nonphosphorylated (on serine-18) \(\alpha_1\)-subunits. Equal protein content for each condition was confirmed using monoclonal antibody 6H (1:1,000). Results are means \(\pm\) SE of at least \(n = 3\) individual experiments. *\(P < 0.05\). Inset: representative immunoblots using McK1 and 6H for control (Con) and insulin-treated samples.

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anism promoting this dephosphorylation in response to insulin appears to require activation of a PI 3-kinase- and PKC-dependent pathway.

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