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Novel Effect of Insulin: Insulin-Stimulated Na^+ Transport Is Mediated by Hydrolysis of Phosphoinositides

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Previous studies showed that insulin stimulation of electrogenic Na^+ transport in renal epithelial cells is mediated by a calcium-dependent signal transduction mechanism. The present study was performed to determine whether the insulin-induced increase in intracellular Ca^{2+} (Ca_i^{2+}) was mediated by hydrolysis of phosphatidylinositol and release of inositol trisphosphate. Experiments were conducted with cultured A6 cells, derived from *Xenopus laevis*, grown on permeable supports. Addition of insulin resulted in 2 to 3 fold increases in inositol trisphosphate and a 50% increase in 1,2 diacylglycerol within 10s, which corresponded to the time-course, previously reported, of insulin stimulated increases in Na^+ transport and Ca_i^{2+}. Further studies showed that aldosterone, previously shown to stimulate an increase in 1,4,5-inositol trisphosphate at onset of the rise in Na^+ transport, also increased DAG levels during the initial phase of stimulation of Na^+ transport. These studies provide the first evidence that a biological response induced by insulin is mediated by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) which results in two products, inositol trisphosphate which causes the release of Ca^{2+} from intracellular stores and 1,2 diacylglycerol. In addition this study provides further support for the proposal that a common signal transduction mechanism mediates electrogenic Na^+ transport by multiple agonists.

Recent studies in this laboratory showed that the action of multiple agonists, including insulin, which stimulate electrogenic Na^+ transport by renal epithelial cells, is mediated by a calcium-dependent mechanism (1-4). Aldosterone, vasopressin and adenosine were found to stimulate the release of Ca^{2+} from intracellular, non-mitochondrial stores via the production of inositol trisphosphate derived from the hydrolysis of phosphatidylinositol. The source of the insulin-induced increase in Ca_i^{2+}, however, was not determined (4). Although some cellular actions of insulin in non-polarized cells are mediated by calcium-dependent processes via increased cellular influx of Ca^{2+} (5-7), calcium release from intracellular stores has not been demonstrated, despite intensive investigation (8-10). This study was therefore, performed to determine whether insulin stimulation of Na^+ transport in renal epithelial cells, like that of other agonists, involves turnover of membrane phosphoinositides and production of inositol trisphosphates and 1,2 diacylglycerol.

MATERIALS AND METHODS

Cultured cells and Na^+ transport. Experiments were performed on a clone of A6 cells derived from the kidney of *Xenopus laevis*. The methods employed for cell culture and measurement of Na^+ transport have recently been reported in detail (2). Since Na^+ transport is measured in cells grown to confluence on porous membranes, the measurement of inositol trisphosphate and DAG were also performed on A6 cells grown to confluence on porous Falcon membranes (Falcon Cell Culture, Beckton Dickenson, Franklin Lakes, N.J.).

Inositol trisphosphate (IP_3). The method used to measure total cellular IP_3 has been reported (2). Cells were grown to confluence on 4.5 cm^2 Falcon membranes. In brief, total IP_3 was measured by high pressure liquid chromatography from cells labeled with myo [^{3}H] inositol (50 μ Ci/ml) for 48 hr in inositol free amphibian Ringer's solution. LiCl was not added to the external bathing solution.

Measurements of 1,2 diacylglycerol (DAG) content. Cells were seeded on Falcon membranes and insulin or aldosterone were added to the basal solution, as indicated in the figure legends, while culture cups were maintained at 26°C. In initial experiments the reaction was stopped with 0.2% SDS added to the apical solution. It seemed likely, however, that under this condition DAG was rapidly degraded because within 1 min after addition of agonist an increase in DAG was observed in some but not in all experiments. To stop the reaction more rapidly, 1 ml of ice cold methanol was added to cell monolayers in subsequent experiments and resulted in the persistent identification of early increases in DAG content after stimulation with agonist. This method was employed in experiments shown.

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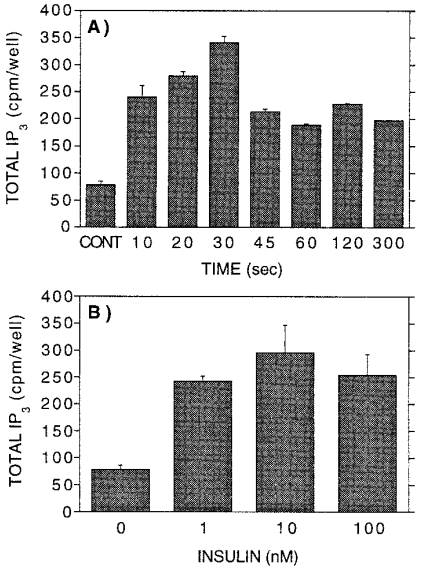

FIG. 1. Insulin stimulation of total inositol trisphosphate (IP_3). A, time-dependent effect of 0.1μM insulin. There were two samples at each time point and values are mean ± range. B, dose-dependent stimulation of IP_3 by insulin. The reaction was stopped at 20S. There were three samples for each concentration. These data are representative of two separate experiments. Values are mean ± SEM.

After brief exposure to ice cold methanol, the broken cell preparation was aspirated several times with a pipettman and then transferred to a glass tube containing 1 ml $CHCl_3$. Following addition of 0.8 ml of H_2O to the membrane, the remaining adherent cells were scraped mechanically and transfered to the same tube containing $CHCl_3$, followed by a second wash with 1 ml methanol. Samples of cell extract were vortexed for 20 to 30s, incubated on ice for 1-2 hrs, vortexed again after addition of 1 ml of $CHCl_3$ and then 1 ml of 0.2 M NaCl, and finally centrifuged for 4 to 5 min at 2000 rpm to permit removal of the lower phase containing lipids. The extract was dried under N_2 and stored under N_2 atmosphere at −70°C until the assay was performed.

Neutral lipids were extracted as previously described (11,12), and DAG content was determined using a modification of the DAG kinase method of Preiss (13).

Reagents. Insulin was obtained from Sigma (St. Louis, MO) and diacylglycerol kinase from Calbiochem, (San Diego, CA). All other reagents were of the highest grade.

RESULTS

Insulin-Stimulated Inositol Trisphosphate (IP_3) Production

Our previous study suggested that the insulin-induced increase in Ca_i^{2+} resulted from degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield IP_3, since Ca^{2+} influx was not apparently increased (4). Total IP_3 was therefore, measured before and after addition of insulin. As shown in Figure 1A, 1 μM insulin caused an increase in IP_3 in a time-course which corresponded to the onset of changes in Ca_i^{2+}, previously reported (4). Levels reached a maximum at 30s and persisted for at least 300s. Subsequently, a dose-response analysis was performed at 10s after addition of various insulin concentrations. Figure 1B demonstrates that 1 nM insulin stimulated a three-fold increase in IP_3. The lack of a progressive rise in IP_3 with higher concentrations of insulin may reflect increased rates of degradation because LiCl was not used to inhibit the metabolism of inositol phosphates or that 10 nM was maximal. The insulin-induced increase in IP_3 implies that Ca^{2+} was released from intracellular stores.

Effect of Insulin on 1,2 Diacylglycerol Production

In addition to an increased production of IP_3 the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates 1,2 diacylglycerol (DAG), which is the natural agonist for protein kinase C activation. Further studies were performed therefore, to determine the effect of insulin on DAG content. Figure 2A demonstrates a rapid increase in DAG content after addition of insulin which reached a maximum level at 10s, followed by a fall to or below the control level. A rapid but transient increase in agonist-stimulated DAG production has been reported to characterize phosphatidylinositide turnover, in contrast to other sources of DAG which are activated more slowly (14). Figure 2B shows that insulin increased DAG content

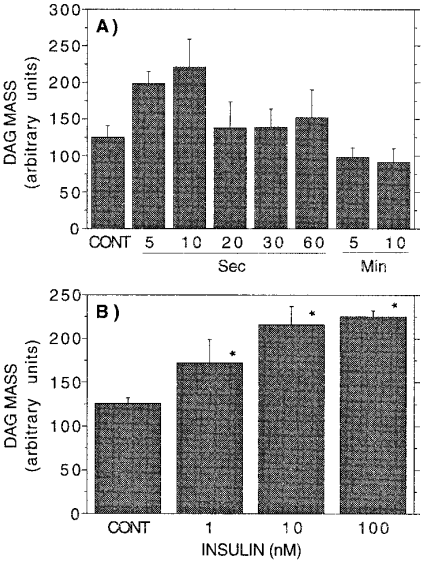

FIG. 2. Effect of insulin on generation of DAG content. A, time-dependent stimulation of DAG content by 1 μM insulin. These data represent one of two separate experiments, each containing two samples at each time point. Values are mean ± range. B, dose-dependent stimulation of DAG content. The reaction was stopped at 10s. There were 5 samples in each group and values are mean ± SEM. The DAG content was determined with Imagequant (3.1) software on a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and are expressed in arbitrary units, representing the integrated area of the ^{32}[P] labeled DAG sample. The symbol * indicates a $p<0.05$ compared to control.

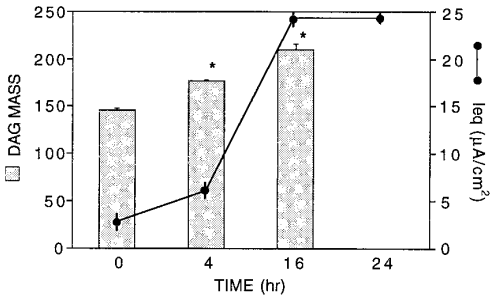

FIG. 3. Effect of aldosterone on Ieq and DAG content. Aldosterone (1μM) was added at 0 time. Ieq was measured sequentially at the times indicated in five samples and values are mean ± SEM. The measure of Ieq was extended to 24 hr to demonstrate that aldosterone stimulated transport was maximal at 16 hr. In the DAG study there were two samples at each time point and values are mean ± range. The symbol * indicates $p < 0.05$ compared to control. The DAG data are taken from one of two separate experiments.

dose-dependently in the range of concentrations reported to stimulate Na^+ transport.

Action of Aldosterone on 1,2 Diacylglycerol Production

If multiple agonists employ the same common signal transduction mechanism for stimulation of Na^+ transport, it can expected that each agonist would also stimulate DAG. Aldosterone-induced Na^+ transport, in contrast to the rapid action of insulin, increases slowly after a delay of about 1 hr to reach a maximum level in cultured A6 cells at about 16 hr (1). Previous studies demonstrated that exposure of A6 cells to aldosterone, which binds to an intracellular receptor, activates a transcription process and results in a transient increase in Ca_i^{2+} due to the action of 1,4,5-inositol trisphosphate after a delay of about 60 min (1). Figure 3 demonstrates an increase in DAG compared to control at 4 hr and at the time of maximal stimulation (16 hr). Together with previous observations these results suggest that insulin and aldosterone share a common signal transduction mechanism in mediating Na^+ transport. To our knowledge this is the first demonstration that DAG generation is stimulated by the action of aldosterone.

DISCUSSION

Insulin stimulates electrogenic Na^+ transport in renal epithelial cells (A6) by activating apical membrane Na^+ channels with the same biophysical characteristics reported after exposure to aldosterone or vasopressin (15). This effect has clinical relevance because insulin-stimulated Na^+ retention in Type II diabetes, and other conditions with chronic hyperinsulinemia, may be causally related to the development of hypertension and atherosclerosis (16). Physiological elevations of insulin are known to increase the absorption of Na^+ in the distal nephron of normal subjects (17). Recent studies performed on cultured renal epithelial cells in this laboratory showed that insulin-stimulated Na^+ transport is mediated by a calcium-dependent mechanism, and suggest that increased levels of Ca^{2+} are derived from intracellular stores (4). This response occurred in the absence, as well as the presence, of millimolar Ca^{2+} in the outside bathing solution. Furthermore, these studies suggested that PKC activation may also mediate insulin-stimulated Na^+ transport because the highly specific PKC antagonist dehydroxychlorpromazine and trifluoperazine inhibited transport dose-dependently in concentrations that approximated the Ki observed with the isolated enzyme. The present study was performed to determine whether insulin-induced Na^+ transport is associated with hydrolysis of PIP_2 to yield inositol trisphosphate, an agonist that releases Ca^{2+} from nonmitochrondial intracellular stores, and DAG, the natural agonist for PKC activation.

This study provides the first demonstration that insulin stimulates hydrolysis of PIP_2 to generate inositol trisphosphate. After exposure of A6 cells to insulin, an increase in the intracellular levels of inositol trisphosphate and DAG, the second product of PIP_2 hydrolysis, were observed in the same time course and in the same dose range that characterize the onset of insulin stimulated Na^+ transport (4). It is known that insulin does stimulate the de novo synthesis of phospholipids and DAG in hepatocytes (10,18), myocytes (7,8), skeletal muscle (19), and adipocytes (6,8,9,20). This reaction results in the generation of phosphatidic acid, phosphatidylinositol, phosphatidyl 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (8). To date, however, insulin-stimulated hydrolysis of PIP_2 to yield water soluble inositol phosphates, inducing 1,4,5-inositol trisphosphate, has not been found in non-polarized insulin-responsive cells (8-10).

A Ca^{2+}-dependent signal mechanism has been reported to mediate insulin-stimulated glucose uptake and oxidation in adipocytes (6,20) and modulation of plasma membrane conductance in heart and smooth muscle cells (5). Since insulin-induced generation of inositol trisphosphate was not observed in these types of cells, it is not surprising that the source of increased Ca_i^{2+} was extracellular Ca^{2+} rather than release from intracellular stores. An increase generation of DAG and activation of PKC have also been demonstrated to mediate insulin-stimulated changes in carbohydrate metabolism in non-polar cells (7,8,19,20). In these studies DAG was shown to be derived from phosphatidic acid. Since insulin stimulation resulted in PIP_2 hydrolysis in A6 cells and DAG levels increased within seconds, our data argue that DAG was generated directly from phosphatidylinositols. Further studies with labeled substrates will be needed to confirm this proposal.

These studies therefore, demonstrate a unique signaling system for insulin that involves phosphatidylinositol turnover, the generation inositol trisphosphate and DAG, and release of Ca^{2+} from intracellular stores in renal epithelial cells with the capacity for electrogenic Na^+ transport. This mechanism is similar to that described for other agonists that stimulate Na^+ transport after binding to surface receptors, such as vasopressin and adenosine, and aldosterone which activates an intracellular receptor. Since the time course of action of aldosterone on electrogenic Na^+ transport is gradual and reaches a maximum only after approximately 16 hours, in contrast to agonists which bind to surface receptors and act rapidly, we explored the affect of aldosterone on intracellular levels of DAG at 4 and 16 hours, when the increase in Na^+ transport is partial and maximal, respectively. The generation of DAG was stimulated at both time points, suggesting that DAG plays a role in mediating the onset of agonist-stimulated Na+ transport even when there are marked differences in the rate of the biological response. Together, these results support the proposal that a common signal transduction mechanism mediates multiple agonists which stimulate electrogenic Na^+ transport.

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