Biologically active lipids promote trafficking and membrane association of Rac1 in insulin-secreting INS 832/13 cells

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McDonald P, Veluthakal R, Kaur H, Kowluru A. Biologically active lipids promote trafficking and membrane association of Rac1 in insulin-secreting INS 832/13 cells. Am J Physiol Cell Physiol 292: C1216–C1220, 2007. First published October 11, 2006; doi:10.1152/ajpcell.00467.2006.—Despite emerging evidence to suggest that glucose-stimulated insulin secretion (GSIS) requires membrane targeting of specific small G proteins (e.g., Rac1), very little is known with regard to the precise mechanisms underlying subcellular trafficking of these proteins in the glucose-stimulated islet β-cell. We previously reported activation of small G proteins by biologically active lipids via potentiation of relevant GDP/GTP exchange activities within the β-cell. Herein, we studied putative regulatory roles for these lipids in the trafficking and membrane association of Rac1 in cell-free preparations derived from INS 832/13 β-cells. Incubation of INS 832/13 cell lysates with polyphosphoinositides (e.g., PIP2), phosphatidic acid, phosphatidylethanolamine, and phosphatidylserine significantly promoted trafficking of cytosolic Rac1 to the membrane fraction. Lysophosphatidic acid, but not lysocephatidylcholine or lysocephatidylethanolamine, also promoted translocation and membrane association of Rac1. Arachidonic acid, diacylglycerol, calcium, and cAMP failed to exert any clear effects on Rac1 translocation to the membrane. Together, our findings provide the first direct evidence in support of our recent hypothesis (Kowluru A. Veluthakal R. Diabetes 54: 3523–3529, 2005), which states that generation of biologically active lipids, known to occur in the glucose-stimulated β-cell, may mediate targeting of Rac1 to the membrane for optimal interaction with its putative effector proteins leading to GSIS.

Acknowledgments

IT IS WELL ESTABLISHED THAT physiological nutrient secretagogues of insulin, such as glucose, do not work via receptor-dependent mechanisms but rather via the generation of soluble mediators, including cyclic nucleotides, calcium ions, and biologically active lipids (17, 22, 26, 27). One such group of mediators consists of hydrolytic products of phospholipids, which are generated by phospholipase A2 (PLA2), phospholipase C (PLC), and phospholipase D (PLD). It has also been demonstrated that such lipids stimulate insulin secretion from adult as well as neonatal rat islets (22). While the exact mechanisms underlying their insulino tropic effects are not well understood, it has been proposed that they might play a regulatory role in stimulus-secretion coupling distal to early plasma membrane events and possibly late in the exocytotic cascade (see Ref. 17 for a review).

Experimental data from different laboratories suggest that some of these “lipid second messengers” may regulate the function of specific G proteins in different cell types. For example, mitogenic lipids such as phosphatidic acid and arachidonic acid display inhibitory effects on guanosine triphosphatase (GTPase)-activating proteins in mouse brain cell extracts (34) and may inhibit GDP/GTP exchange activity of purified smg p21 (10). In neutrophils, arachidonic acid appears to promote GTP binding (21). It has also been shown that arachidonic acid and phosphatidic acid accelerate, and may mediate, the generation of the superoxide anion by NADPH oxidase in human neutrophils (21); the stimulation by arachidonic acid appears to require a pertussis toxin-sensitive G protein in addition to one or more small G proteins. Along these lines, we provided the first evidence to indicate regulation of G proteins in islet subcellular fractions by phospholipase-derived lipid mediators of insulin secretion (14). In those studies, using cell-free preparations, we have demonstrated stimulation of GTP binding as well as inhibition of GTPase activities by specific biologically active lipids. On the basis of this evidence, we concluded that the insulino tropic nature of lipids might, in part, be due to their ability to maintain G proteins in their active GTP-bound conformation by enhancing GTP binding and decreasing GTP hydrolysis (14).

More recent studies from our laboratory have identified that Rac1, a member of the Rho subfamily of G proteins, plays a significant contributory role in glucose-stimulated insulin secretion (GSIS) (19, 20). Furthermore, we have reported localization of, and regulation by, Rac1 regulatory proteins, such as the GDP dissociation inhibitor (e.g., Rho-GDI) in GSIS (19). The principal objective of the current studies is to examine putative contributory roles, if any, for the known second messengers of insulin secretion (e.g., lipids, cAMP, or calcium) in promoting membrane association of Rac1 in INS 832/13 cells, which we have demonstrated to be a necessary step for GSIS (19, 20). Using INS 832/13 cell lysates, we demonstrate herein that specific lipids, but not calcium or cAMP, promote translocation and membrane association of Rac1.

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MATERIALS AND METHODS

Materials. Arachidonic acid, arachidonic acid methyl ester, 1-α-phosphatidic acid, 1-α-phosphatidylcholine, phospho-1-serine, 1-α-lysophosphatidic acid, diacylglycerol (DAG), cAMP, SDS, and deoxycholic acid were purchased from Sigma (St. Louis, MO). 1-α-Phosphatidyl-1-mylo-inositol-4,5-bisphosphate (PIP₂) was obtained from Calbiochem (San Diego, CA). Antisera directed against Rac1 and Rho-GDI were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Insulin-secreting cells. INS 832/13 cells were provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC). They were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μg 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The medium was changed twice weekly and cells were trypsinized and subcloned weekly.

Translocation and membrane association of Rac1. This was determined according to a method described earlier (19, 20). In brief, INS 832/13 cells were rinsed briefly in phosphate buffer saline, scraped, and spun at 100 g for 5 min to remove the medium. The cellular pellet was homogenized in an isotonic medium consisting of 250 mM mannoti, 70 mM sucrose, 5 mM HEPES, pH 7.4, containing 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail. The cell lysates were then incubated with various lipids (dissolved in appropriate solvents) for 15 min at 37°C. After incubation, the lysates were spun at 105,000 g for 60 min (Beckman Ultima TL-100) to separate the total membrane (pellet) and the cytosolic (soluble) fractions (3, 19, 20). The purity of the membrane fraction was determined by the relative enrichment of E-cadherin, a membrane marker protein, as we described earlier (19). Proteins from each of these fractions were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti-Rac1 or anti-Rho-GDI antisera followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution) for 1 h. Immune complexes were detected using enhanced chemiluminescence (ECL) kit. Intensity of the protein bands was quantitated by densitometry, as we described in Refs. 3, 19, and 20.

Protein assay. Protein concentration in the membrane and cytosolic fractions was determined by the dye-binding method of Bradford using BSA as the standard.

Statistical analyses of experimental data. Statistical significance of differences was determined by ANOVA. A P value < 0.05 was considered significant.

RESULTS

Extant studies from our laboratory and those of others have demonstrated localization of G protein regulatory factors such as guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs) in clonal β-cells and normal rat islets (19, 29, 32). Of immediate relevance to the current studies are recent observations from our laboratory to directly implicate GDIs in GSIS (19). In the aforementioned studies, we also hypothesized that glucose-mediated ADP-ribosylation factor-6 (ARF-6)-dependent generation of intracellular second messengers, such as polyphosphoinositides (e.g., PIP₂) or phosphatidic acid, might facilitate dissociation of the Rac1/GDI complex followed by translocation of Rac1 to the membranous fraction (19). Along these lines, we have also demonstrated that Rac1 activation and membrane association are essential for GSIS (19, 20). With these observations in mind, we designed and utilized an in vitro assay to quantitate potential regulatory role(s) for exogenously added lipids, calcium, or cAMP in the dissociation of the Rac1/GDI complex and subsequent membrane association of Rac1 in INS 832/13 cell lysates.

Data in Fig. 1 indicate nearly equal amounts of Rac1 in membranous and cytosolic fractions derived from INS 832/13 cells. However, a brief (15 min; see MATERIALS AND METHODS for additional details) incubation of INS 832/13 cell lysates with PIP₂ (30 μM) resulted in translocation of the majority of the cytosolic Rac1 to the membranous fraction.

In the next series of studies, we determined potential regulatory roles for various phospholipids (e.g., phosphatidic acid, phosphatidylserine, or phosphatidylcholine; 30 μM each) in facilitating the membrane association of Rac1 under the conditions described in Fig. 1. Data in Fig. 2 indicate that all three lipids, namely phosphatidic acid, phosphatidylserine, and phosphatidylcholine, significantly promoted membrane association of Rac1.

As indicated above, we have recently demonstrated that the majority of Rac1 remains complexed with GDI in the cytosolic compartment of the β-cell (19). Therefore, we examined changes, if any, in the subcellular distribution (membrane vs. cytosolic) of GDI in INS 832/13 cell lysates incubated with various lipids, described under Fig. 2. Under these conditions, the majority of the GDI appeared to remain predominantly in the cytosolic fraction as evidenced by Western blotting, although we observed a modest increase in the membranous association of GDI in the presence of lipids (Fig. 3).

To further determine the specificity of the lipid effects, we determined the relative abundance of Rac1 in the membrane fraction following incubation of INS 832/13 cell lysates with various lysophospholipids (e.g., lysophosphatidic acid, lysophosphatidylcholine, lysophosphatidylserine; 30 μM each). Data in Table 1 indicate a significant increase in membrane association of Rac1 in the presence of lysophosphatic acid but not lysophosphatidylcholine or lysophosphatidylserine. Also, we observed no significant effects of either arachidonic acid or arachidonic acid methyl ester (its inactive analog; 30 μM each) on translocation of Rac1. These values represented 100 ± 25 and 100 ± 10% of control in the presence of arachidonic acid and arachidonic acid methyl ester, respectively (n = 3 determinations in each case; additional data not shown). These data appear to suggest specificity with regard to specific effects of different biologically active lipids on Rac1 translocation to the membrane fraction.
We next determined the membrane association of Rac1 in INS 832/13 cell lysates incubated with various detergents (e.g., SDS or sodium deoxycholate; 50 \( \mu \)M each) to rule out the possibility that the effects seen in the presence of specific lipids are not due to their “detergent-like” properties. Data in Fig. 4 suggest no significant effects of either of these detergents on membrane-to-cytosol ratios of Rac1 abundance in INS 832/13 cells. Together, these data provide further support for specificity of various lipids on Rac1 translocation to the membranous fraction and exclude any possibility of detergent action of these lipids in mediating such effects.

In the final set of studies, we examined potential roles of calcium (50 \( \mu \)M) and cAMP (100 \( \mu \)M) on Rac1 translocation.
under the current experimental conditions. Data in Table 1 indicate no significant effects of either calcium or cAMP on membrane association of Rac1. Moreover, DAG (30 μM) failed to significantly affect Rac1 translocation and association with the membrane under these conditions. These values represented 100 ± 43% of control in the presence of DAG (n = 3, P = not significant; additional data not shown). Taken together, these data appear to suggest specific effects of biologically active lipids, but not calcium, cAMP, or DAG, on trafficking and membrane association of Rac1 in insulin-secreting INS 832/13 cells (see below).

**DISCUSSION**

Studies from multiple laboratories, including our own, have provided convincing experimental evidence to implicate the Rho subfamily of G proteins (e.g., Rac1 and Cdc42) in GSIS (2, 3, 6–20, 23–25, 29, 32). Typically, these G proteins remain in cytosol in their GDP-bound, inactive conformation as complexes with their regulatory proteins, such as the GDIs (4, 7, 19, 30, 31). Along these lines, we have recently provided the first evidence to demonstrate a direct regulatory role for GDI in GSIS (19). In these studies, we have demonstrated that glucose promotes translocation of Rac1 to the membrane fraction in clonal β- (INS and βTC3) cell preparations as well as in normal rat islets. On the basis of these data, we hypothesized that Rac1 translocation to the membrane in the glucose-stimulated β-cell may be mediated via biologically active lipid moieties generated by the direct actions of glucose on various phospholipases indigenous to the β-cell (22). This study, therefore, represents a logical extension to our recent attempts to understand mechanisms underlying glucose-mediated activation of specific G proteins endogenous to the islet β-cell. Our principle findings are 1) PIP2, phosphatidic acid, phosphatidylserine, and phosphatidylinositol specifically promote membrane targeting of Rac1 in cell-free preparations of INS cells; 2) lysophosphatidic acid, but not lysophosphatidylinositol or lysophosphatidylserine, is effective in promoting the translocation to the membrane; 3) arachidonic acid, an insulinotropic fatty acid, has no demonstrable effects on membrane association of Rac1 under these conditions; 4) regulatory effects of lipids, as in 1, may not be due to detergent properties of these lipids, since SDS and deoxycholic acid failed to exert any effects on membrane association of Rac1; and 5) neither calcium, cAMP, nor DAG elicited any effects on membrane association of Rac1. On the basis of these data, we conclude that generation of specific lipids via glucose-induced activation of lipid-metabolizing enzymes might represent a regulatory step in the cascade of events leading to membrane association of Rac1 and subsequent hitherto unidentified steps leading to GSIS.

Previous studies from our laboratory (14) have demonstrated regulation, by lipid messengers, of endogenous G proteins in various subcellular fractions (e.g., secretory granules) isolated from normal rat islets. For example, we have reported stimulation of GTP binding and inhibition of GTP hydrolysis by insulinotropic lipids in subcellular fractions of the pancreatic islet. On the basis of those data, we proposed that insulinotropic effects of these lipids may, in part, be due to their ability to retain putative G proteins in their GTP-bound active conformation. Again, the major significance of the aforementioned studies (14) and the current investigation may reside in the fact that physiological secretagogues of insulin release do not work via receptor-dependent mechanisms but via the generation of soluble second messengers. Earlier studies have documented the presence of PLA2 and PLD in pancreatic islets. In addition, it has been shown that phospholipid hydrolytic products of PLA2 and PLD stimulate insulin secretion from isolated islets (see Ref. 22 for a review). Data from our earlier studies (14) and the current investigation may thus provide a unifying hypothesis for the insulin-releasing abilities of the lipids, in that they might elicit direct regulatory effects on specific G proteins (e.g., Rac1) whose activation and membrane association are required for GSIS to occur.

Earlier observations in other cell types provide further support to our current findings in insulin-secreting INS 832/13 cells. For example, using human neutrophil cytosolic preparations, Chuang et al. (5) provided evidence to suggest dissociation of Rac1/GDI complexes by biologically active lipids. These studies have demonstrated most noticeable effects by arachidonic acid, phosphatidic acid, and phosphatidylinositol. The concentrations of lipids used in our current studies are similar to those employed by Chuang et al. It is also important to note that the concentrations of lipids used in the current investigation are well within the range reported to occur in the glucose-stimulated islet β-cells (Refs. 14, 15, and 22 and references therein). Moreover, in earlier studies, we have been able to demonstrate significant regulatory effects of these lipids (in the concentration range tested herein) on GTP binding and hydrolytic activities in subcellular fractions derived from insulin-secreting cells (14). It is for these reasons that we believe the current findings are physiologically significant.

It is important to note that the majority of our current findings are in agreement with observations by Chuang et al. (5), the only exception being arachidonic acid, which did not exert any clear effects on translocation of Rac1 to the membrane in INS cells. Using Swiss 3T3 fibroblasts, Fleming et al. (8) demonstrated significant translocation of Cdc42, but not Rac1, RhoB, or Rho-GDI, in the presence of lysophosphatidic acid. Effects of lysophosphatidic acid on Rac1 translocation in Swiss 3T3 fibroblasts are in contrast to our observations in INS cells, in which membrane association of Rac1 was significantly promoted by lysophosphatidic acid. More recent studies by Ugolev et al. (33) in artificial liposomal systems have suggested significant Rac1/GDI complex dissociating effects of anionic but not neutral phospholipids. Together, these studies appear to further support our overall conclusions that biologically active lipids promote membrane association of Rac1, albeit the specificity of lipids appears to differ in each study.

Last, using PC3 human carcinoma cells, Price et al. (28) recently reported calcium-dependent activation of Rac1, presumably mediated via the activation of calcium-dependent activation of a conventional protein kinase C. These investigators demonstrated that a forced increase in intracellular calcium concentrations or protein kinase C activation results in the phosphorylation of Rho-GDI, which induces dissociation of Rac1 from the Rac1/GDI complex, thereby enabling Rac1 to translocate to the membrane. However, in our current experimental conditions, we failed to see any clear effects of exogenously provided calcium, cAMP, or DAG on Rac1 association with the membrane. Additional studies are needed to conclusively demonstrate whether Rho-GDI undergoes phosphoryla-
tion in the islet β-cell by a protein kinase and whether such a phosphorylation step facilitates dissociation of the Rac1/GDI complex and targeting of the released Rac1 to the membrane fraction.

In conclusion, our current studies provide the first evidence to indicate that specific biologically active lipids, known to be generated within the glucose-stimulated β-cell, may mediate targeting of Rac1 to the membrane for interaction with its putative effector proteins, leading to GSIS. Further studies are needed to elucidate the precise mechanisms through which certain biologically active lipids mediate translocation and membrane association of Rac1 while others lack the specificity in inducing such an effect. Additional studies are needed to conclusively demonstrate whether glucose-induced generation of some of these lipids (e.g., PIP2 or phosphatidic acid) require ARF-6 activation in the islet β-cell, as we originally proposed in Ref. 19.

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