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Regulatory roles for Tiam1, a guanine nucleotide exchange factor for Rac1, in glucose-stimulated insulin secretion in pancreatic β-cells

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Abstract

Using various biochemical, pharmacological and molecular biological approaches, we have recently reported regulatory roles for Rac1, a small G-protein, in glucose-stimulated insulin secretion [GSIS]. However, little is understood with respect to localization of, and regulation by, specific regulatory factors of Rac1 in GSIS. Herein, we investigated regulatory roles for Tiam1, a specific nucleotide exchange factor [GEF] for Rac1, in GSIS in pancreatic β-cells. Western blot analysis indicated that Tiam1 is predominantly cytosolic in distribution. NSC23766, a specific inhibitor of Tiam1-mediated activation of Rac1, markedly attenuated glucose-, but not KCl-induced insulin secretion in INS 832/13 cells and normal rat islets. Further, NSC23766 significantly reduced glucose-induced activation [i.e., GTP-bound form] and membrane association of Rac1 in INS 832/13 cells and rat islets. Moreover, siRNA-mediated knock-down of Tiam1 markedly inhibited glucose-induced membrane trafficking and activation of Rac1 in INS 832/13 cells. Interestingly, however, in contrast to the inhibitory effects of NSC23766, Tiam1 gene depletion potentiated GSIS in these cells; such a potentiation of GSIS was sensitive to extracellular calcium. Together, our studies present the first evidence for a regulatory role for Tiam1/Rac1-sensitive signaling step in GSIS. They also provide evidence for the existence of a potential Rac1/Tiam1-independent, but calcium-sensitive component for GSIS in these cells.

Keywords

NSC23766; Tiam1; Rac1; Pancreatic β-cells; Glucose-stimulated insulin secretion; calcium-induced insulin secretion

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1. Introduction

Insulin secretion from pancreatic β-cells is regulated principally by the ambient concentrations of glucose. However, the molecular and cellular mechanisms underlying the stimulus-secretion coupling of GSIS from the pancreatic β-cells remain only partially understood. It is well established that the signaling steps involved in GSIS from the β-cell requires a well regulated trafficking of insulin-laden secretory granules for their docking and fusion with the plasma membrane [1-3]. Emerging evidence also suggests that such cellular events are under the fine control of small G-proteins, which have been implicated in cytoskeletal remodeling to facilitate granule movement [1,4]. Original observations from multiple laboratories, including our own, have convincingly demonstrated critical involvement of small G-proteins, such as Rac1, Cdc42, Rap1 and the ADP-ribosylation factor-6 [ARF-6] in GSIS from normal rat islets, human islets, and clonal β-cell preparations [1,5-24]. Such conclusions were drawn primarily based on data from experiments utilizing: [a] inhibitors of requisite post-translational modifications [e.g., prenylation, carboxylmethylation and acylation] of certain G-proteins; [b] Clostridial toxins, which monoglucosylate and inactivate specific G-proteins; and [c] gene manipulation experiments involving the dominant negative and constitutively active mutants of these G-proteins [1,14]. Further, more recent evidence from our laboratory demonstrated that overexpression of an inactive mutant of the regulatory α-subunit of protein prenyl transferase results in a marked attenuation of GSIS in insulin-secreting INS 832/13 cells [25]. Together, these data afford support to our original hypothesis that activation of small G-proteins [e.g., Rac1 and Cdc42] is a necessary step in signaling events leading to GSIS.

In a manner akin to the heterotrimeric G-proteins, small G-proteins cycle between their GDP-bound [inactive] state and GTP-bound [active] conformations, which are tightly regulated by various G-protein regulatory factors [GRFs]. At least three types of GRFs have been described for small G-proteins [4,17]. The GDP/GTP exchange factors [GEFs] stimulate the conversion of the GDP-bound form to the GTP-bound form; the GDP-dissociation inhibitors [GDIs] elicit inhibition of this signaling step by preventing the dissociation of GDP from the candidate G-proteins; and the GTPase-activating proteins facilitate conversion of the GTP-bound to the GDP-bound form by activating the GTPase activity intrinsic to respective G-proteins. Despite a large body of evidence on the localization of the GRFs in multiple cell types, very little is understood with regard to localization and putative regulation, by GRFs, of GSIS in isolated β-cells. Along these lines, we have recently reported immunological localization of Rho-GDI in normal rat islets and INS 832/13 cells [17]. Further, using siRNA targeted against Rho-GDI, we have been able to demonstrate a negative modulatory role for Rho-GDI in GSIS from pancreatic β-cells [17]. These data further support our original hypothesis for potential regulatory roles for Rac1 and its GRFs [e.g., GDI] in GSIS. Along these lines, recent studies by Nevins and Thurmond indicated novel roles for caveolin-1 as the GDI for Cdc42, a small G-protein, which has also been implicated in GSIS [26].

In the context of potential regulation of Rac1, multiple GEFs have been identified in other cell types. These constitute the diffuse B cell lymphoma [Dbl] family of GEFs, including Trio and Tiam1 [27,28]. Recently, Zheng *et al* have developed NSC23766, which is a soluble first generation small molecule inhibitor of Tiam1-mediated activation of Rac1 [29]. These investigators have reported significant inhibition of Rac1-GTP-loading by NSC23766 without significantly affecting the GTP-loading onto other small G-proteins including Cdc42 and Rho A. Under these conditions, NSC23766 also attenuated cell proliferation induced by Tiam1, which is a Rac1-specific GEF. Based on these data, Zheng and coworkers concluded that NSC23766 represents a specific inhibitor of Tiam1-mediated activation of Rac1 [29,30]. Several other laboratories have utilized NSC23766 since then to decipher potential contributory roles for Tiam1/Rac1 signaling pathway in cellular functions [31-41]. With this in mind, and as a logical extension to our ongoing studies, which provided a compelling evidence for key

regulatory roles for Rac1 in GSIS, we undertook the current investigation to determine potential role of Tiam1-mediated Rac1 signaling steps in GSIS in INS 832/13 cells [referred to as β-cells throughout the manuscript]. We accomplished the above objective by two independent approaches; first through the use of NSC23766 to impede the functional activation of Rac1 mediated by Tiam1, and second *via* the siRNA-mediated knock-down of endogenous Tiam1. Our findings implicate key regulatory roles for Tiam1 in GSIS.

2. Materials and Methods

2.1. Materials

Mouse monoclonal antibody directed against Rac1 was purchased from BD Bioscience [San Jose, CA]. Affinity purified rabbit polyclonal antibody against Tiam1 and NSC23766 were purchased from Calbiochem [San Diego, CA]. The rat insulin ELISA kit was purchased from American Laboratory Products Co [Windham, NH]. Small interfering RNA [siRNA], a pool of 3 target specific 20-25 nt designed to knock-down gene expression of Tiam1 and scrambled-siRNA [negative control] were purchased from Santa Cruz Biotechnology, Inc [Santa Cruz, CA].

2.2. Isolation of rat islets

Islets were isolated from normal Sprague-Dawley rats [150-250g, Harlan, Indianapolis, Indiana] using the collagenase digestion method as described previously [10,17]

2.3. Insulin-secreting cells

INS 832/13 were kindly provided by Dr. Chris Newgard [Duke University School of Medicine, Durham, NC] and were cultured as described previously [17,19].

2.4. Transfection with siRNA

For these studies, β-cells were plated in 24 well plates at 30-40% confluence a day before transfection. siRNAs were mixed with HiPerfect transfection reagent obtained from Qiagen [Valencia, CA] and cells were transfected with either Tiam1 or scrambled-siRNA at a final concentration of 100 nM. Comparison of transfection experiments were done against control [non-transfected cells], mock transfected cells [reagent alone] or cells transfected with scrambled-siRNA [negative control]. The efficiency of the siRNA transfection was verified in each study by immunoblot analysis of Tiam1.

2.5. Insulin release studies

Control or Tiam1-depleted β-cells were treated with either diluent alone or NSC23766 [0-50 μM] and were cultured overnight in low glucose media. Cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous absence or presence of NSC23766 as indicated in the text. For determination of rapid phase and slow phase GSIS, cells were cultured in 24-well plates and were stimulated with either 5 or 20 mM glucose for 10 minutes, and the medium removed for the determination of rapid secretion event. Afterward, the cells were incubated with fresh medium containing the same stimuli for an additional 30 min for assessment of slow phase secretion [19]. Insulin released into the medium was quantitated by ELISA [17].

2.6. Insulin content measurement

Insulin content measurement was carried out as we described earlier [21]. Briefly, β-cells treated with either diluent alone or NSC23766 were extracted overnight in acid/ethanol mixture [77% absolute ethanol, 22% water and 1% HCl (v/v)] at 4°C. After 20 h, the extracts were centrifuged, and the amount of insulin was quantitated by ELISA as above.

2.7. Immunological detection and subcellular distribution of Tiam1 in β-cells

The total soluble [supernatant] or membrane [pellet] fractions isolated from lysates by single-step centrifugation at 105,000*g* for 90 min were separated by SDS-PAGE and the resolved proteins were transferred to a nitrocellulose membrane. Blots were then probed with antibody raised against Tiam1 [1:500 dilution; overnight] and then incubated with secondary antibody conjugated to horseradish peroxidase [HRP]. Immune complexes were detected using the enhanced chemiluminescence [ECL] kit.

2.8. Translocation and membrane association of Rac1

The degree of translocation of cytosolic Rac1 to the membrane fraction was assessed as we described previously [6,17,19]. In brief, control or Tiam1-depleted β-cells were treated with either diluent alone or NSC23766 [20 μM] and cultured overnight in low glucose-containing media. The next day, cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous presence of either diluent or NSC23766. After the incubation period, cells were homogenized by sonication and subjected to a single-step centrifugation at 105,000*g* for 60 min. Total membrane [pellet] and soluble [supernatant] fractions were separated and used for the determination of relative abundance of Rac1 in these fractions by Western blotting.

2.9. Rac1 activation assay

The relative degree of Rac1 activation [i.e., GTP-bound form] was determined using a Rac1 activation assay [Cytoskeleton, Inc., Denver, CO] as we described in [6,17]. In brief, β-cells were treated with either diluent alone or NSC23766 [20 μM] or β-cells transfected with either Tiam1 or scrambled-siRNA were cultured overnight in low glucose media. Cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous presence of either NSC23766 or diluent. Lysates [1-2mg protein/ml] were clarified by centrifugation for 5 min at 4,800*g*, and PAK-PBD [p21-activated kinase-p21-binding domain] beads [20 μl] were added to the supernatant. The mixture was then rotated for 1 h at 4°C and pelleted by centrifugation at 4,000*g* for 3 min. This pellet was washed once with lysis buffer followed by a rinse in wash buffer [25 mM Tris, pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, and 150 mM EDTA] and was then reconstituted in Laemmli buffer. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, and the relative abundance of Rac1 was determined by Western blotting method as described above.

2.10. Metabolic cell viability determinations

The β-cells were seeded at a density of 1×10^6 cells/ml in round-bottomed 96-well plates and then treated with diluent or NSC23766 [0-50 μM; 18 h as indicated in the text]. Cell viability was determined by a colorimetric assay [at 550-690 nm], using 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium bromide [MTT] [Roche Applied Science, Indianapolis, IN], which measures the reduction of MTT into the blue formazan product, by metabolically active cells [42].

2.11. Other assays

Protein concentration in β-cell subcellular fractions was quantitated according to Bradford using bovine serum albumin as the standard [43].

2.12. Statistical analyses of the experimental data

The statistical significance of the differences between the experimental conditions was determined by ANOVA. A p value < 0.05 was considered significant.

3. Results

3.1. Immunological localization of Tiam1 in pancreatic β-cells

Recent evidence from our laboratory implicated glucose-mediated Rac1 activation in GSIS. We also reported regulation of GSIS by Rho-GDI, a GDP-dissociation inhibitor for Rac1 in isolated β-cells [17]. Since the current studies are aimed at understanding potential regulation of GSIS by Tiam1, a Rac1-specific GEF, we examined, at the outset, localization of Tiam1 in pancreatic β-cells. For this, we used an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to an amino acid sequence mapping at the C terminus of Tiam1. Western blot analysis of β-cell lysates indicated that the Tiam1 antibody recognizes a single band of ~200 kDa, which corresponded to the known molecular mass of Tiam1 [Figure 1; panel A]. Further studies revealed that Tiam1 is predominantly cytosolic in its distribution [Figure 1; panel B]. These data are compatible with reports of localization of Tiam1 within the cytosolic compartment in other cell types [27,28,44,45].

3.2. NSC23766 specifically inhibits GTP loading onto Rac1, but not to Cdc42 or Rho

As stated above, NSC23766 has been used to specifically inhibit Tiam1-mediated activation of Rac1 in multiple cell types. Herein, we verified specificity of NSC23766 to inhibit activation of Rac1 in pancreatic β-cells *in vitro*. For this purpose, β-cell lysates were incubated with GTPγS in the absence or presence of NSC23766 [500 μM]. Rac1, Cdc42 and Rho activation assay were carried out [see Methods for additional details]. Figure 2 [panels A and D] demonstrate that NSC23766 specifically inhibited Rac1-GTP formation, but not Cdc42-GTP [Figure 2; panels B and D] or Rho-GTP formation [Figure 2; panels C and D]; these findings are compatible with those by Zheng *et al* [29,30].

3.3. NSC23766 exerts no significant effects on total protein, total insulin contents or metabolic cell viability in isolated β-cells

We next verified potential alterations, if any, in total protein and insulin contents in β-cells following incubation with NSC23766 [20 μM]. We found that the total protein content represented 28.6 ± 2.14 and 33.2 ± 1.59 μg in diluent-and NSC23766-treated cells, respectively [n=3 determinations in each case]. Likewise, the insulin content represented 73.7 ± 21.1 and 100.9 ± 25.5 pg/μg protein in diluent-and NSC23766-treated cells, respectively [n=3 measurements in each case]. It is also important to note that in Western blot analyses, we were unable to detect any significant differences in the expression of either Rac1 or Tiam1 in cells treated with NSC23766 [additional data not shown]. We also quantitated the metabolic cell viability in β-cells treated with NSC23766 to rule out the possibility that it might exert cytotoxic effects following inhibition of Tiam1-mediated activation of Rac1 in these cells. Our data indicate no differences in the degree of metabolic cell viability in cells treated with NSC23766 [0-50 μM] compared to those incubated in the presence of diluent alone. The cell viability rates represented 107.80 ± 3.30 percent of control in NSC23766-treated cells [not significant vs. diluent-treated cells; n=3 determinations in each case]. It must be also noted that, in a limited number of studies we observed clear changes in the cytoskeleton as evidenced by cell rounding in β-cells exposed to NSC23766 [additional data not shown]. This might reflect potential alterations in the cytoskeletal proteins, which are expected to occur following inactivation of specific small molecular weight G-proteins [e.g., Rac1]. Together, our findings suggest that NSC23766 exerts no cytotoxic effects in isolated β-cells as evidenced by no clear effects of NSC23766 on total protein content, insulin content, Rac1 and Tiam1 expression as well as metabolic cell viability in β-cells.

3.4. NSC23766 inhibits GSIS in insulin-secreting cells

We next determined the effect of NSC23766 on GSIS from β-cells. In these studies we observed that NSC23766 [20 μM] had minimal effect on basal insulin secretion seen in the presence of 5 mM glucose [i.e., 6.36 ± 0.30 ng/ml and 5.79 ± 0.17 ng/ml in control and NSC23766-treated cells, respectively; n=3 determinations]. Data in Figure 3 demonstrate a significant [~50%] inhibition of GSIS was demonstrable in β-cells incubated overnight with [1 μM] NSC23766. We observed a much larger [nearly 80%] inhibition of GSIS in the presence of a higher concentration [20 μM] of NSC23766. A more pronounced inhibition of GSIS seen in the presence of higher concentrations of NSC23766 may not be due to its cytotoxicity since we failed to detect any significant effects of NSC23766 at this concentration on total cellular protein, insulin content or metabolic cell viability [see above]. It should be noted that shorter periods of incubation [e.g., 4 h even at 50 μM] failed to significantly affect GSIS [data not shown] presumably due to relatively slower rates of uptake of this compound as reported in other cell types [29,46]. Hence, in the subsequent experiments, we adopted the incubation protocols as described under Figure 3. Furthermore, NSC23766 [25 μM; overnight] inhibited GSIS in normal rat islets albeit to a varying degree. For example, the degree of inhibition by NSC23766 of GSIS in isolated islets ranged from ~ 17% [16.03 ± 0.58 vs. 13.38 ± 1.31 ng/ml] to ~ 60% [16.51 ± 1.08 vs. 6.72 ± 0.51 ng/ml; n = 3-4 independent experiments; additional data not shown]. Together, these data demonstrate that pharmacological inhibition of Tiam1-mediated activation of Rac1 results in inhibition of GSIS in normal rat islets and clonal β-cells.

3.5. Inhibition of Tiam1 leads to impairment in glucose-mediated trafficking and membrane association of Rac1

Recent findings from our laboratory have demonstrated glucose-stimulated translocation and membrane association of Rac1 in normal rat islets and clonal β-cell preparations [17,25]. Herein, we determined the ability of glucose to promote membrane association of Rac1 in β-cells [INS 832/13 cells and rat islets] exposed to the diluent alone or NSC23766. Incubation of β-cells with stimulatory glucose concentrations significantly increased [~ 2 fold; Figure 4; panel A, lane 2 vs. lane 4] the abundance of Rac1 in the membrane fraction; compatible with our earlier observations [17,25]. However, the ability of glucose to promote trafficking and membrane association of Rac1 was nullified by treatment of these cells with [20 μM] NSC23766 [Figure 4; panels A and B]. Similar results were observed when rat islets incubated with stimulatory glucose concentrations. The relative abundance of Rac1 in the membrane fraction was 1.865 ± 0.74 fold higher in glucose-treated islets compared to those exposed to basal glucose. Exposure of normal rat islets to NSC23766 markedly reduced the ability of glucose to promote trafficking of Rac1 to the membrane fraction [e.g., 0.738 ± 0.21 fold increase in NSC23766-treated [20 μM; 18 h] islets vs.1.865 ± 0.74 fold in diluent-treated islets]. These data support our hypothesis that inhibition of Tiam1-mediated activation of Rac1 leads to inhibition of glucose-induced membrane association of Rac1 [Figure 4] culminating in inhibition of GSIS under these conditions [Figure 3].

3.6. Inhibition of Tiam1 activation leads to attenuation of glucose-induced activation of Rac1

Next set of experiments were conducted to determine if inhibition of Tiam1 by NSC23766 attenuates glucose-induced functional activation of Rac1, under conditions in which it inhibited GSIS [Figure 3] and association of Rac1 with the membrane fraction [Figure 4]. Treatment of β-cells with stimulatory concentration of glucose [20 mM] significantly increased Rac1 activation as evidenced by data from the PAK-PBD pull down assay which determines the amount of GTP-bound Rac1 [Figure 5; panel A lanes 1 vs. 2]. However, ability of glucose to promote Rac1 activation [~ 2 fold, Figure 5; panel B] was significantly attenuated by NSC23766 [Figure 5; panel A lanes 3 vs. 4]. It must be noted that NSC23766 inconsistently, but modestly increased Rac1 activation under basal glucose conditions [Figure 5; panel A lanes

1 vs. 3]. However, such increases did not reach statistical significance [additional data not shown]. Data from multiple experiments further confirmed these observations [Figure 5; panel B]. Together, these findings suggest that inhibition of Tiam1 by NSC23766 impedes the ability of glucose to promote activation [Figure 5] and membrane association [Figure 4] of Rac1 leading to inhibition of GSIS [Figure 3].

3.7. Tiam1-mediated Rac1 activation is not necessary for insulin secretion elicited by a membrane-depolarizing concentration of KCl

We next determined potential regulatory effects of Tiam1-mediated Rac1 activation on KCl-induced insulin secretion from β-cells. Our data indicate that KCl-induced insulin secretion is completely resistant to NSC23766 [i.e., KCl-induced insulin secretion represented 90.7 ± 21.11 and 81.3 ± 4.47 ng/ml in diluent-and NSC23766-treated cells, respectively; n=3 independent experiments]. These findings suggest that signaling steps involving Tiam1-mediated activation of Rac1 is relevant for glucose- [Figure 3], but not KCl-induced insulin secretion from β-cells. These findings support our recent observations indicating no significant roles for Rac1 in KCl-induced insulin secretion. For example, using a dominant negative mutant of Rac1 [N17Rac1], we recently reported that Rac1 activation may not be necessary for insulin secretion demonstrable in the presence of a membrane depolarizing concentration of KCl [19]. We have further confirmed these observations by utilizing an inactive mutant of the regulatory α-subunit of farnesyl/geranylgeranyl transferase, which inhibits the requisite prenylation of small G-proteins, including Rac1 [25]. Together, these findings provide conclusive evidence to suggest that Tiam1-mediated Rac1 activation is not necessary for KCl-induced insulin secretion.

3.8. Paradoxical potentiation of GSIS in β-cells in which expression of endogenous Tiam1 is knocked-down *via* the siRNA approach

Since above observations suggested that pharmacological inhibition of Tiam1-Rac1 interaction lead to functional inactivation of Rac1 and inhibition of GSIS, we undertook a study to determine GSIS in β-cells in which endogenous expression of Tiam1 is reduced by siRNA targeted against Tiam1. Figure 6 [panel A] represents a Western blot depicting a significant reduction [nearly 60%] in the expression of Tiam1 in β-cells transfected with the siRNA targeted against Tiam1, when compared to either non-transfected, mock or scrambled-siRNA transfected cells. Under these conditions, we observed a significant potentiation [~ 2 fold] of GSIS in cells in which Tiam1 expression was knocked-down [Figure 6; panel B, data expressed as incremental response to stimulatory glucose concentration]. No major differences in GSIS were observed between the cells transfected with either mock or scrambled-siRNA [as a negative control; Figure 6; panel B bar 1 and 2]. It should be noted that the total insulin content in control, mock-transfected, scrambled-siRNA or Tiam1-siRNA-transfected cells remained unchanged in low glucose [5 mM]-treated cells. Those values represented 18.5 ± 0.82 pg/μg, 19.4 ± 1.49 pg/μg, 15.6 ± 0.90 pg/μg and 16.6 ± 0.77 pg/μg in control, mock-transfected, scrambled siRNA-transfected and Tiam1 siRNA-transfected cells, respectively [n=3 measurements in each case]. Together, these data demonstrate that while Tiam1-mediated activation of Rac1 is necessary for GSIS [e.g., data from NSC23766 studies]; data from knock-down experiments indicate that Tiam1 might play additional modulatory role[s] in the events leading to GSIS [see below].

We next quantitated the rapid and slow phases of GSIS in control β-cells or in cells in which Tiam1 expression is knocked-down using approaches described under Figure 6. Time-dependent GSIS was assessed under static incubation conditions using a protocol we described earlier [19], which quantifies GSIS during the rapid [0-10 min] or slow [11-45 min] secretion under static incubation conditions. Data in Figure 7 indicate that potentiation of GSIS in Tiam1-depleted cells can be seen only during the slow phase of secretion [Figure 7; lanes 4-6], since

the relative degrees of GSIS from the mock, scrambled or Tiam1-depleted β-cells remained unchanged during the early or rapid phase [Figure 7; lanes 1-3].

3.9. Despite potentiation of GSIS, glucose-induced membrane association and activation of Rac1 is inhibited in Tiam1-depleted β-cells

To rule out the possibility that glucose might still be able to translocate to the membrane fraction and activate Rac1 in Tiam1-depleted cells, we assessed the ability of glucose to activate Rac1 and stimulate Rac1 translocation to the membrane fraction in mock, scrambled and cells in which Tiam1 is compromised *via* the siRNA transfection. We used the protocols described under Figure 4. Data in Figure 8 demonstrate that a stimulatory concentration of glucose facilitates the trafficking and membrane association of Rac1 in cells transfected with scrambled-siRNA [Figure 8; panel A, lanes 2 vs. 4]. However, the ability of glucose to promote membrane association of Rac1 was negated completely in cells in which endogenous levels of Tiam1 were depleted [Figure 8; panel A, lanes 6 and 8]. Data from multiple experiments are given in panel B of Figure 8.

Additional studies were carried out to assess the ability of glucose to activate Rac1 [Rac1-GTP configuration] in cells in which endogenous levels of Tiam1 were depleted. Treatment of β-cells with stimulatory concentration of glucose [20 mM] significantly increased Rac1 activation as evidenced by data from the PAK-PBD pull down assay which determines the amount of GTP-bound Rac1 in mock or scrambled-siRNA transfected cells, whereas cells in which Tiam1 was knocked-down, glucose failed to activate Rac1 [Figure 8; panel C]. It should also be noted that the relative abundance of active Rac1 in mock, scrambled or Tiam1-depleted cells remained unchanged under basal glucose [5 mM] conditions. Such values represented 97.8 ± 4.24 and 92.3 ± 1.80 percent of mock-transfected cells in scrambled siRNA and Tiam1 siRNA transfected cells, respectively [n=3 determinations in each case]. Together, based on the data depicted in Figures 4 and 8, we conclude that Tiam1-mediated activation is necessary for glucose to promote trafficking and membrane association of Rac1 and activation of Rac1 in isolated β-cells. Data in Figure 8 also reveal that potentiation of GSIS in Tiam1-depleted cells might be regulated by additional factors/proteins, which appear to be different from Rac1 and Tiam1 [see below].

3.10. Potentiation of GSIS in Tiam1-depleted β-cells is sensitive to extracellular calcium

In the last set of experiments, we verified sensitivity of the GSIS potentiating effect in Tiam1-depleted cell to extracellular calcium. To determine this, we quantitated GSIS in cells transfected with mock, scrambled or Tiam1-siRNA and exposed to regular media or a media in which extracellular calcium is removed according to the protocol we described earlier [47]. First, compatible with data described in Figure 6, a significant potentiation of GSIS was seen in cells following Tiam1 depletion [Figure 9; lanes 1 or 2 vs. 3]. Second, as expected, GSIS from control cells was inhibited significantly following removal of extracellular calcium from the medium [Figure 9; bars 1 vs. 4]. Similar degree of inhibition was demonstrable in cells transfected with scrambled-siRNA [used as negative control; Figure 9; bars 2 vs. 5]. Third, even though there was a modest potentiation of GSIS in Tiam1 depleted cells in calcium-free conditions [Figure 9 lanes 4 or 5 vs. 6], such differences between mock or scrambled vs. Tiam1 depleted cells did not reach statistical significance. Based on these findings, we conclude that the potentiated secretion in Tiam1-depleted cells is sensitive to extracellular calcium. Taken together, our findings suggest that Tiam1-Rac1 cross talk represents one of the signaling steps involved in GSIS. They also suggest that additional Rac1/Tiam-1 independent and calcium-sensitive mechanism might underlie GSIS in pancreatic β-cells [see Discussion].

4. Discussion

Using various biochemical, immunological, physiological and molecular biological approaches, previous studies have conclusively demonstrated that at least two members of the Rho subfamily of G-proteins, namely Cdc42 and Rac1, play significant contributory roles in GSIS [6-10,14,15,17,19,22-25]. One of the specific objectives of this current study was to determine the localization of, and potential regulation by, Tiam1, a Rac1-specific GEF, in pancreatic β-cells. Salient features of the current study are: [i] NSC23766, a specific inhibitor of Tiam1-Rac1 signaling pathway, inhibited glucose-, but not KCl-induced insulin secretion from isolated β-cells; [ii] NSC23766 also inhibited the ability of glucose to activate and translocate Rac1 to the membrane fraction; [iii] glucose-induced membrane association and activation of Rac1 was also reduced significantly in cells in which endogenous expression of Tiam1 is reduced *via* siRNA transfection suggesting that Tiam1 might represent a GEF for Rac1 in β-cells; and [iv] siRNA-mediated depletion of endogenous Tiam1 expression potentiated GSIS in an extracellular calcium-dependent manner.

Recent studies from the laboratory of Zheng *et al* suggested that NSC23766 specifically inhibits Tiam1-induced activation of Rac1, but not Cdc42 or Rho. For example, these investigators have determined the specificity of Rac1 inhibition by NSC23766 *via* structure-based virtual screening of several compounds that fit into a surface groove of Rac1 known to be critical for Tiam1 binding [29,30]. Further, using *in vitro* experimental approaches, these researchers have also demonstrated specific inhibition of Tiam1-mediated activation of Rac1 by NSC23766, without significantly affecting the activation of Cdc42 or Rho by their respective GEFs [29, 30]. In addition, under *in vivo* conditions, NSC23766 effectively blocked serum or platelet derived growth factor-induced Rac1 activation and subsequent lamellipodia formation without affecting the activation of Cdc42 or Rho [29,30]. Based on these findings, and other compelling evidence, these investigators have concluded that NSC23766 represents a novel small molecule inhibitor that could be used to specifically study the involvement of Rac1 activation in cellular function. Recently, several other investigators have also used NSC23766 to further decipher putative regulatory roles for Tiam1/Rac1 signaling pathway in cellular function [31-41]. Herein, using NSC23766, we have been able to present conclusive evidence to suggest that it specifically inhibits GTP loading onto Rac1, but not Cdc42 and Rho and such a signaling step in necessary for GSIS to occur.

Our findings indicate that inhibition of Tiam1-mediated activation of Rac1 results in attenuation of glucose-, but not KCl-induced insulin secretion from pancreatic β-cells. These data imply that such a signaling step may not be necessary for events leading to KCl-stimulated insulin secretion. Such a formulation is compatible with earlier findings suggesting resistance of KCl-induced insulin secretion from normal rat islets and clonal β-cell preparations to inhibitors of isoprenoid biosynthesis such as lovastatin [20,21]. Further, using an inactive mutant of Rac1 [i.e., N17Rac1], we have demonstrated that Rac1 activation maybe necessary for glucose-, but not KCl-induced insulin secretion [19]. More recently, using an inactive mutant of the regulatory α-subunit of prenyl transferases, we have demonstrated that geranylgeranylation of Rho G-proteins [e.g., Rac1] may not be necessary for KCl-induced insulin secretion [25]. Together, these findings appear to implicate a regulatory role for Rac1 only in glucose-, but not KCl-induced insulin secretion. Data from our laboratory and those from Thurmond and coworkers further suggest that activation of Cdc42, another member of Rho subfamily of GTPases, is not required for calcium-induced secretion [22,48]. In this context, recent studies by Lawrence and Birnbaum [18] implicated activation of ARF-6 in calcium-induced secretion from clonal β-[MIN-6] cells and normal rat islets. Previous data from our laboratory have suggested that while the carboxylmethylation, a requisite post-translational modification for G-protein activation, of Cdc42 is stimulated by glucose, but not KCl, the carboxylmethylation of Rap1 was stimulated by both glucose and KCl [15,47]. We

have shown that glucose-induced carboxylmethylation of specific γ subunits of trimeric G-proteins, which is necessary for functional activation of trimeric GTPases, also required the presence of extracellular calcium [47]. Thus, at least three potential candidates [e.g., ARF-6, Rap or γ subunits of trimeric G-proteins] might mediate KCl-[or calcium] induced secretion. Additional studies are required to precisely identify the putative G-protein[s] responsible for calcium-induced secretion.

What then are the mechanisms [or signaling steps] underlying glucose-mediated activation of Tiam1/Rac1 signaling steps that could contribute toward GSIS?. Mertens *et al* [27] recently described potential mechanisms for the intracellular regulation of Tiam1 including regulation of its expression under specific experimental conditions; regulation of its function by intramolecular inhibition, by changes in intracellular localization or by post-translational modifications and interaction with other proteins. While some of these possibilities could potentially contribute toward glucose-induced activation of Tiam1/Rac1 in the β-cell, most compelling evidence comes from the ability of glucose to regulate these functions *via* the generation of intracellular second messengers [e.g., biologically active lipids]. For example, recent studies by Fleming and coworkers [49] have provided direct evidence to suggest that local concentrations of signaling lipids and the net intracellular concentrations of cytosolic inositol phosphates play critical modulatory roles for the regulation of Tiam1/Rac1 function *in vivo*. Such a model would also support our recent data [including current] on glucose-mediated activation of Tiam1/Rac1 and subsequent events leading to GSIS in β-cells. For example, we previously demonstrated that biologically active lipids mediate activation of GDP/GTP exchange, presumably *via* the intermediacy of GEFs, in subcellular fractions isolated from normal rat islets [50] and promote membrane association of Rac1 [51]. Together, these data appear to strengthen our proposal for a direct role[s] for Tiam1/Rac1 in the sequence of events leading to GSIS, presumably involving biologically-active lipid second messengers of insulin secretion.

A growing body of evidence also implicates lysophospholipids, specifically lysophosphatidic acid [LPA] in the regulation of Tiam1 function. For example, Fleming *et al* [49] have demonstrated a protein kinase C-sensitive, LPA-induced threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts. In a more recent study, Van Leeuwen and coworkers [52,53] have reported LPA-mediated activation of Rac1 through the intermediacy of Tiam1. These investigators have also demonstrated that overexpression of LPA receptor in B103 neuroblastoma cells resulted in the activation of Rac1 leading to cell spreading, lamellipodia formation culminating in increased cell migration. More importantly, LPA-mediated activation of Rac1 was not demonstrable in cells lacking Tiam1. Based on these data, these researchers have concluded that Tiam1-mediated activation of Rac1 is necessary for LPA's effects in these cells. The above findings have immediate relevance to our current findings for potential involvement of Tiam1-mediated Rac1 activation in GSIS. Moreover, we have recently reported that LPA, but not lysophosphatidylserine or lysophosphatidylcholine, markedly stimulated trafficking and membrane association of Rac1 in INS 832/13 cells [51]. It remains to be seen, however, if LPA also mediates the conversion of GDP-bound inactive form of Rac1 to its GTP-bound active conformation *via* activation of Tiam1 in β-cells.

Interestingly, data from our current study suggest that siRNA-mediated inhibition of Tiam1 expression leads to a paradoxical increase in GSIS. Potential mechanisms underlying such an effect remain unknown at the present time. We offer the following explanations. First, it is likely that Tiam1, by itself, or Tiam1-activated Rac1 might control activities of putative transcriptional factors that might play negative modulatory roles in GSIS; regulation of cellular function by such factors [e.g., STAT and c-myc] has been described in other cell types [54, 55]. This needs to be verified in the β-cell. Second, inhibition of Tiam1 leads to activation of other GEFs for Rac1 [e.g., vav, Trio; 56 for a review]. Indeed, such a redundant activation

mechanism for Rac1 following Tiam1 depletion has been described recently by Strumane and coworkers in T-lymphoma cells [57]. However, it is unlikely since we failed to detect glucose-mediated membrane association and activation of Rac1 in Tiam1 depleted cells [see Results]. Third, since the potentiation of GSIS seen following Tiam1 is a calcium-sensitive step [Figure 10], and it is likely that potentiated secretion might be due to activation of other G-proteins which require calcium for optimal activation [see above].

Lastly, recent evidence appears to suggest a significant cross talk between Tiam1, Rac1, and nm23 H1 [a tumor suppressor gene] in various cellular signal transduction processes [58]. It has also been shown that nm23H1 translocates to the membrane fraction, which then recruits Tiam1 to further regulate Rac1 function [59]. In this context, published evidence from our laboratory suggests a link between islet endogenous nm23H1/nucleoside diphosphate kinases and endogenous G-protein activation [16,60-62]. Further, we recently reported that overexpression of wild type nm23H1, but not its histidine kinase-deficient mutant, significantly potentiated GSIS in insulin secreting cells [63]. It also appears that cytosolic nm23H1 translocates to the membrane fraction in a glucose-stimulated β-cell [unpublished observations from our lab]. Studies to identify potential mechanisms including cross talk between Tiam1, Rac1 and nm23H1 in the context of GSIS are in progress in our laboratory. In conclusion, we demonstrate herein a regulatory role for Tiam1/Rac1-sensitive signaling steps in GSIS. We also provide evidence for the existence of a potential Rac1/Tiam1-independent, but calcium-sensitive component for GSIS in these cells.

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Abbreviations

ARF-6
: ADP-ribosylation factor-6

G-proteins
: Guanosine triphosphate-binding proteins

GDI
: GDP-dissociation inhibitor

GEF
: Guanine nucleotide exchange factor

GRFs
: G-protein regulatory factors

GSIS
: Glucose-stimulated insulin secretion

Tiam1
: T-lymphoma invasion and metastasis1

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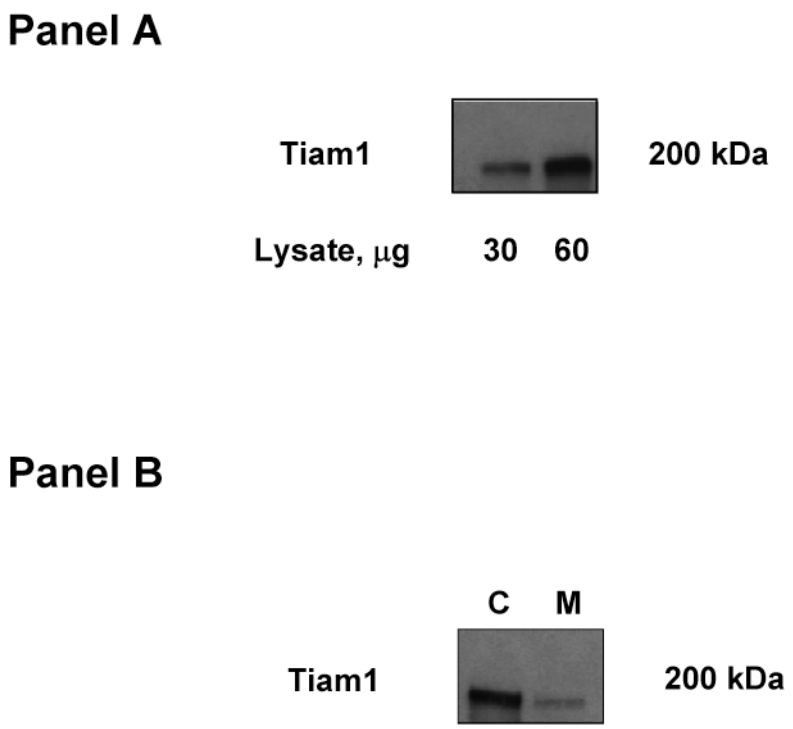

Figure 1. Tiam1 is present in pancreatic β-cells

Panel A: The β-cell lysates [30 or 60 μg protein as indicated] were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then blocked and incubated with an antibody directed against Tiam1 [1:500 dilution; overnight] followed by incubation with HRP-conjugated secondary antibody. Immune complexes were detected using ECL kit. A representative blot from 3 experiments yielding similar results is shown.

Panel B: The β-cell lysates were separated into soluble cytosolic and total membrane fractions by single step centrifugation method [see Methods]. Equal amount of protein [50 μg] from each fraction were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked and incubated with antibody raised against Tiam1 [1:500 dilutions; overnight] followed by incubation with HRP-conjugated secondary antibody. Immune complexes were detected using ECL kit. A representative blot from 3 experiments yielding similar results is shown.

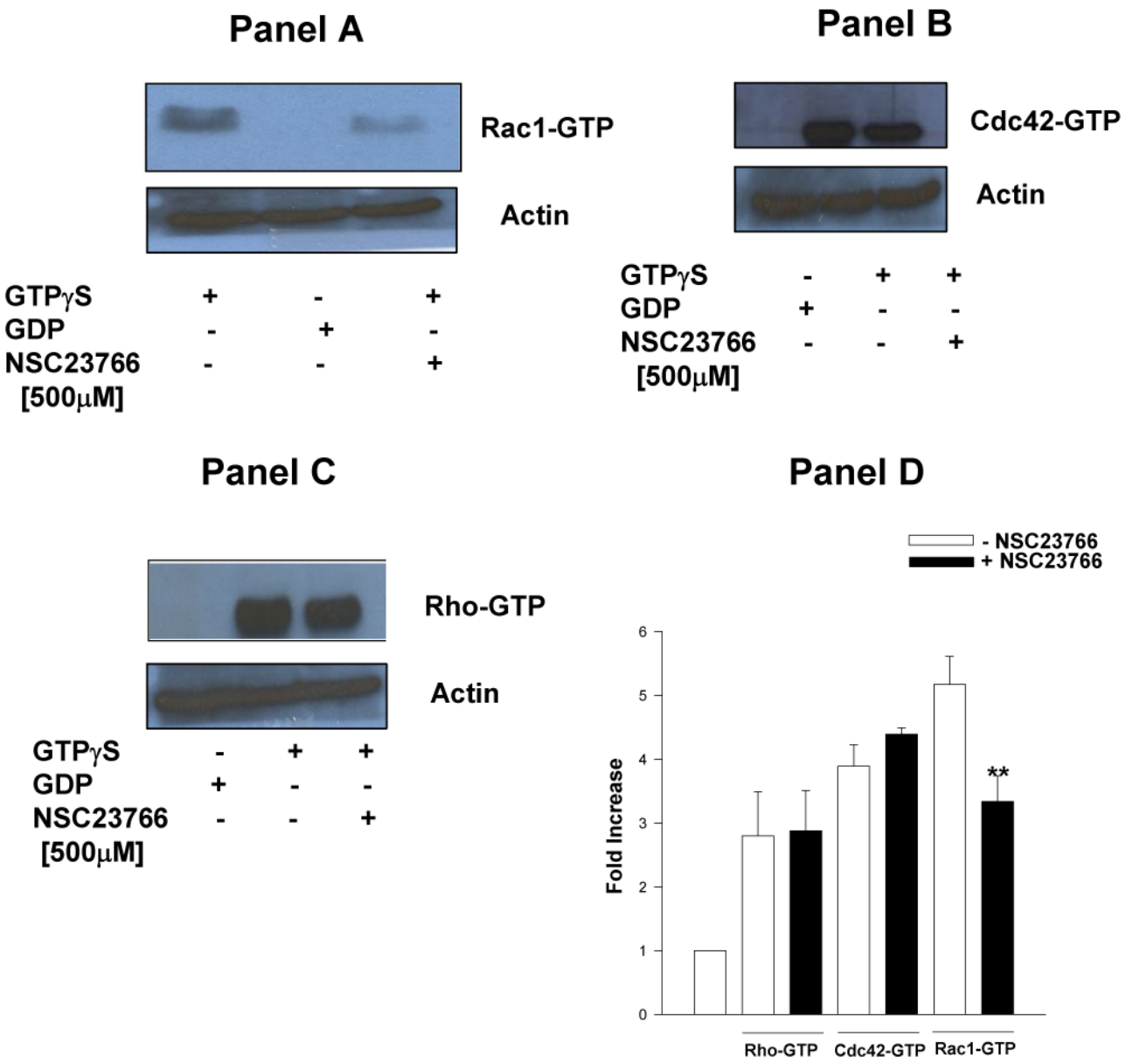

Figure 2. NSC23766 specifically activate Rac1, but not Cdc42 or Rho in pancreatic islet β-cell

Lysates from pancreatic β-cells were incubated with either GDP or GTPγS [200 μM for 10 min] along with diluent or NSC23766 as indicated in the figure. The relative amounts of activated Rac1 [i.e., Rac-GTP; **Panel A**], activated Cdc42 [i.e., Cdc42-GTP; **Panel B**] or activated Rho [Rho-GTP; **Panel C**] were determined by a pull-down assay using PAK-PBD [see Methods]. The proteins thus obtained were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, then probed for Rac1, Cdc42 or Rho. Immune complexes were identified using an ECL kit as described in Methods. A representative blot from three experiments is shown here. Densitometric analysis of the ratio of actin:Rho-GTP, Cdc42-GTP or Rac1-GTP were carried out and [**Panel D**] data are means ± SEM from three different experiments and expressed as fold increase. **$p < 0.05$ vs. diluent treated cells.

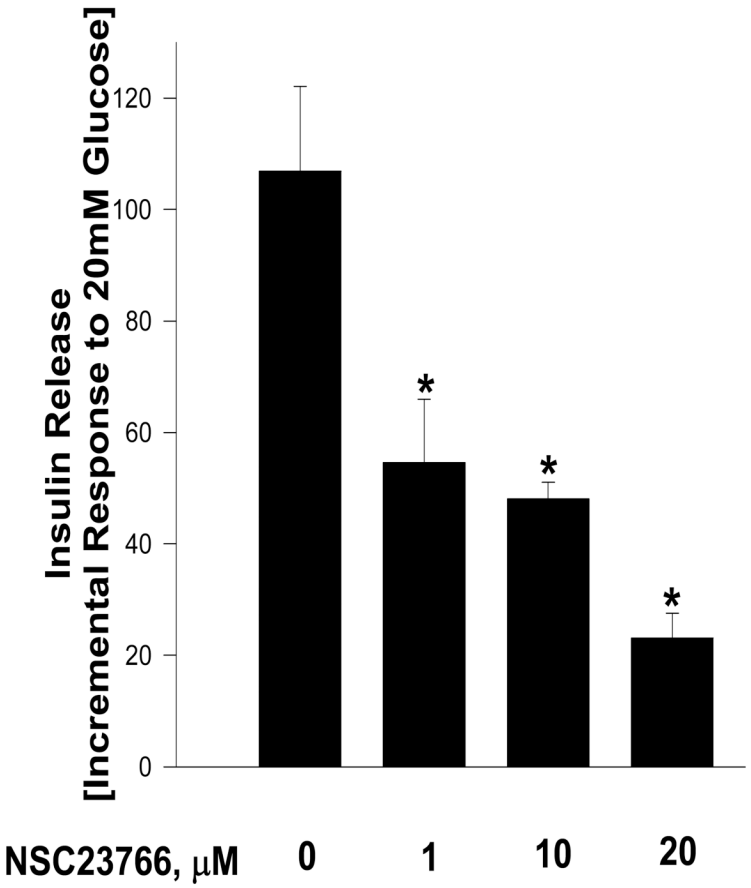

Figure 3. NSC23766 markedly reduces GSIS from β-cells in a concentration-dependent manner

Pancreatic β-cells were treated with either diluent alone or NSC23766 [0-20 μM] as indicated in the figure, and cultured overnight in low glucose media. Cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous presence of either NSC23766 or diluent. Insulin released into the medium was quantitated by ELISA. Data are expressed as incremental response to 20 mM glucose and are means ± SEM from three independent experiments. * represents $p < 0.05$ vs 20 mM glucose alone.

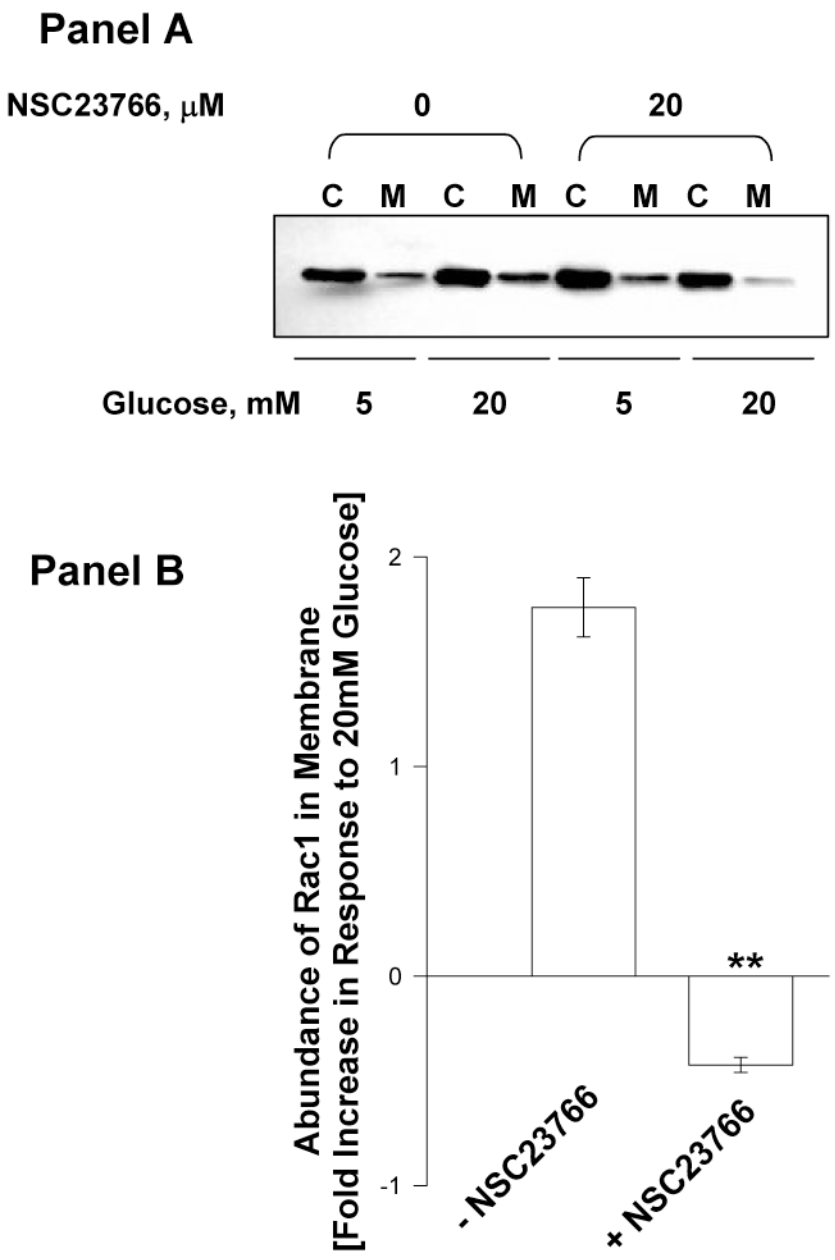

Figure 4. NSC23766 markedly attenuates the ability of glucose to promote translocation of Rac1 to the membrane in β-cells

Panel A: Pancreatic β-cells were treated with either diluent alone or NSC23766 [20 μM] as indicated in the figure, and cultured overnight in low glucose media. Cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous presence of NSC23766 or diluent. Lysates were separated into soluble cytosolic and total membrane pellet fractions by a single step centrifugation method [see Methods]. Proteins from cytosolic [represented as C] and membrane fraction [represented as M] isolated from β-cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked and incubated with antibody raised against Rac1 [1:1000 dilutions; 1h] followed by incubation with HRP-conjugated secondary antibody.

Immune complexes were detected using an ECL kit. A representative blot from 3 experiments yielding similar results is shown.

Panel B: Intensity of the protein bands was quantitated by densitometry. Data are means ± SEM from three different experiments and expressed as fold increase in the membrane fraction. **$p < 0.05$ vs. diluent treated cells challenged with stimulatory glucose concentration alone [20 mM].

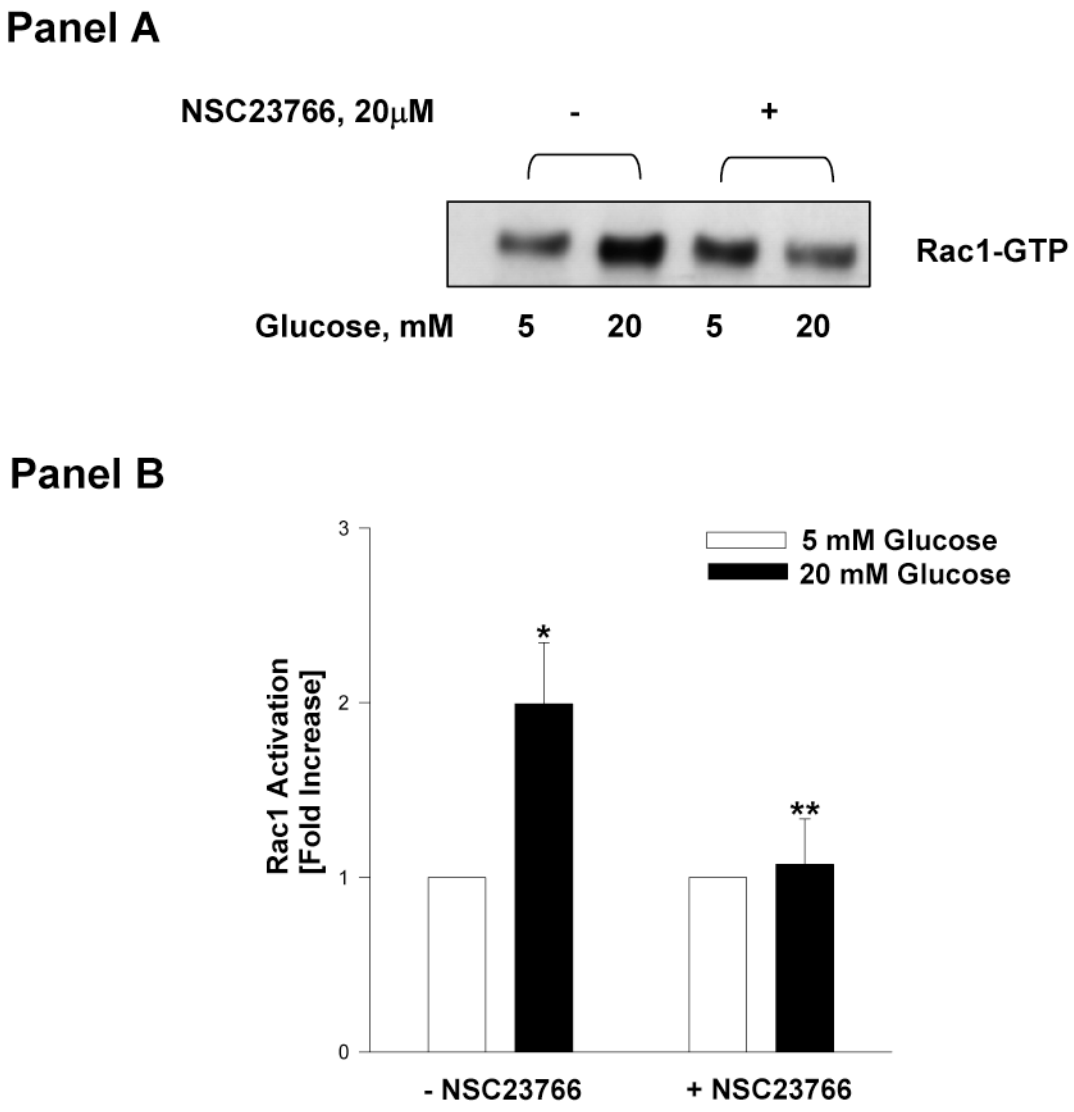

Figure 5. NSC23766 markedly inhibits Rac1 activation by glucose in β-cells

Panel A: Pancreatic β-cells were incubated with either diluent alone or NSC23766 [20 μM] as indicated in the figure, and cultured overnight in low glucose media. Cells were further incubated in the presence of either low [5 mM] or high [20 mM] glucose for 30 min at 37°C in the continuous presence of NSC23766 or diluent. The relative amounts of activated Rac1 [i.e., Rac-GTP] were determined by a pull-down assay [see Methods]. The proteins thus obtained were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, then probed for Rac1. Immune complexes were identified using an ECL kit as described in Methods. A representative blot from three experiments is shown here.

Panel B: Intensity of the protein bands was quantitated by densitometry. Data are means ± SEM from three different experiments and expressed as fold increase. *$p < 0.05$ vs. basal glucose concentration [5 mM] and **$p < 0.05$ vs. stimulatory glucose concentration alone [20 mM].

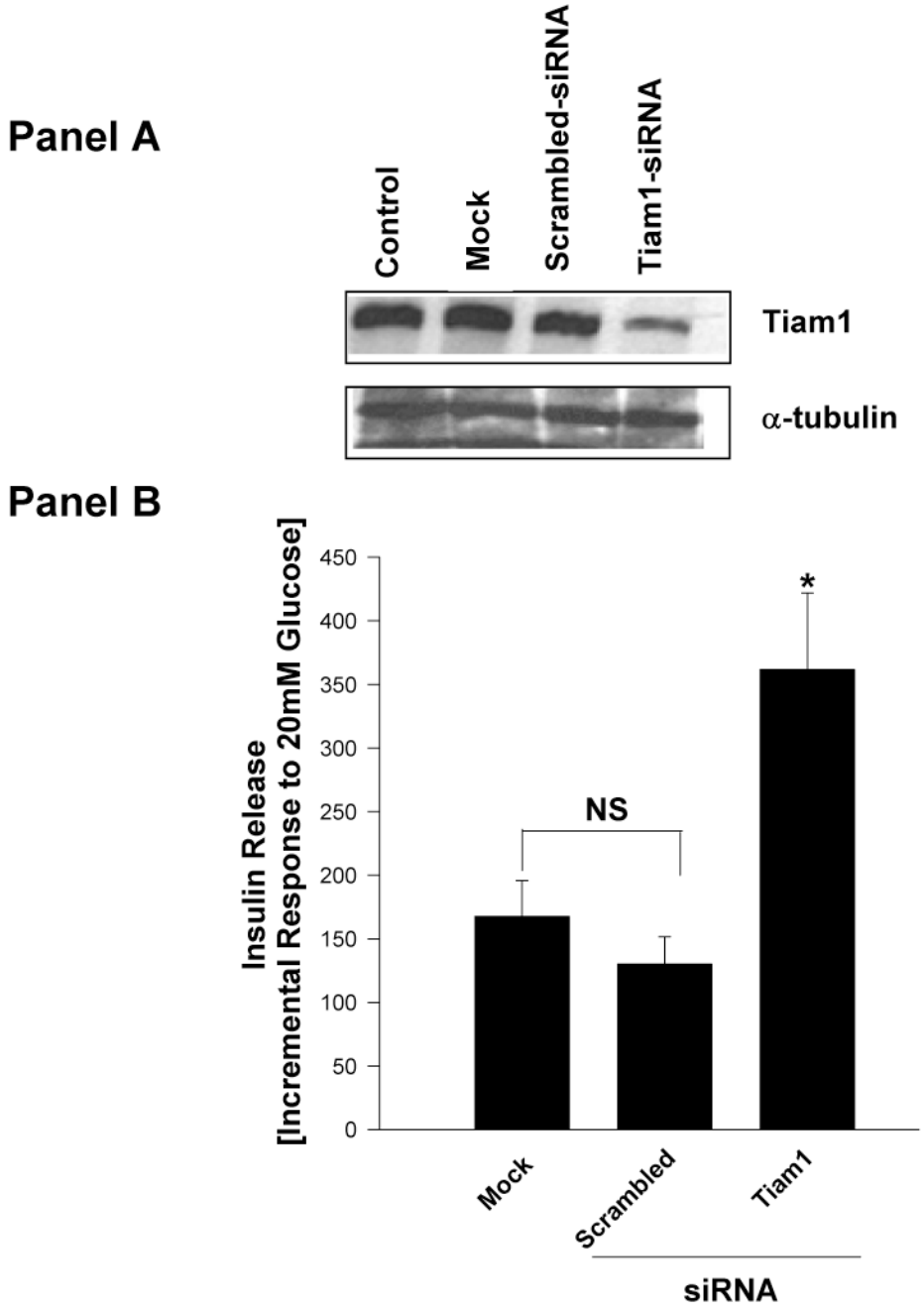

Figure 6. Transfection of siRNA targeted against Tiam1 potentiates GSIS from pancreatic β-cell

Panel A: Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 48 h, the expression of Tiam1 was determined in the lysate protein by Western blotting. A representative blot from three experiments is shown here.

Panel B: Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 24 h, the cells were further incubated in the presence of low glucose media overnight. Cells were further incubated in the presence of either

low [5 mM] glucose [20 mM] for 30 min at 37°C. Insulin released into the medium was quantitated by ELISA. Data are expressed as incremental response to 20 mM glucose and are means ± SEM from three independent experiments. * represents $p < 0.05$ vs mock or scrambled-siRNA transfected cells and NS= Non significant.

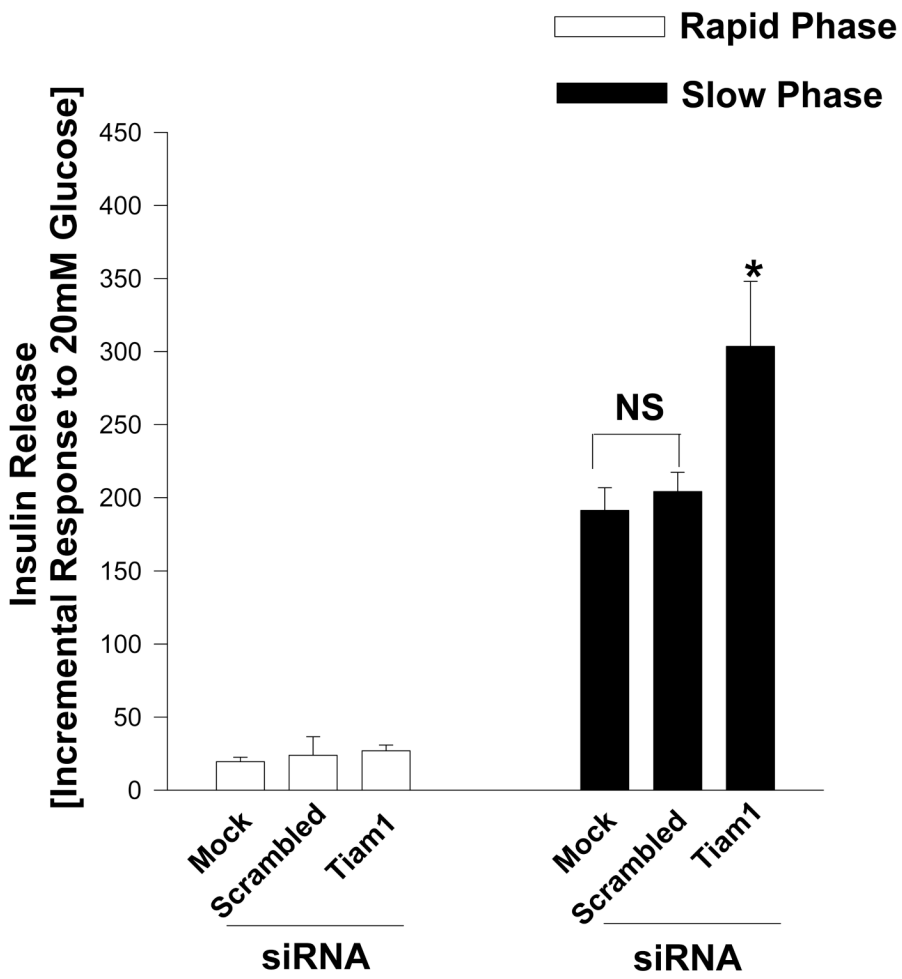

Figure 7. siRNA-mediated knock-down of Tiam1 potentiates slow, but not rapid phase of GSIS from pancreatic β-cell

Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 24 h, the cells were further incubated in the presence of low glucose media overnight. Cells were further incubated in the presence of either low [5 mM] or [20 mM] glucose for 30 min at 37°C. After 10 minutes of incubation, the medium was removed for the determination of the early secretion event. The cells were incubated further with same stimuli for additional 35 min for the assessment of the slow-phase secretion. Insulin released into the medium was quantitated by ELISA. Data are expressed as incremental response to 20 mM glucose and are means ± SEM from three independent experiments. * represents $p < 0.05$ vs mock or scrambled-siRNA transfected cells and NS= Non significant.

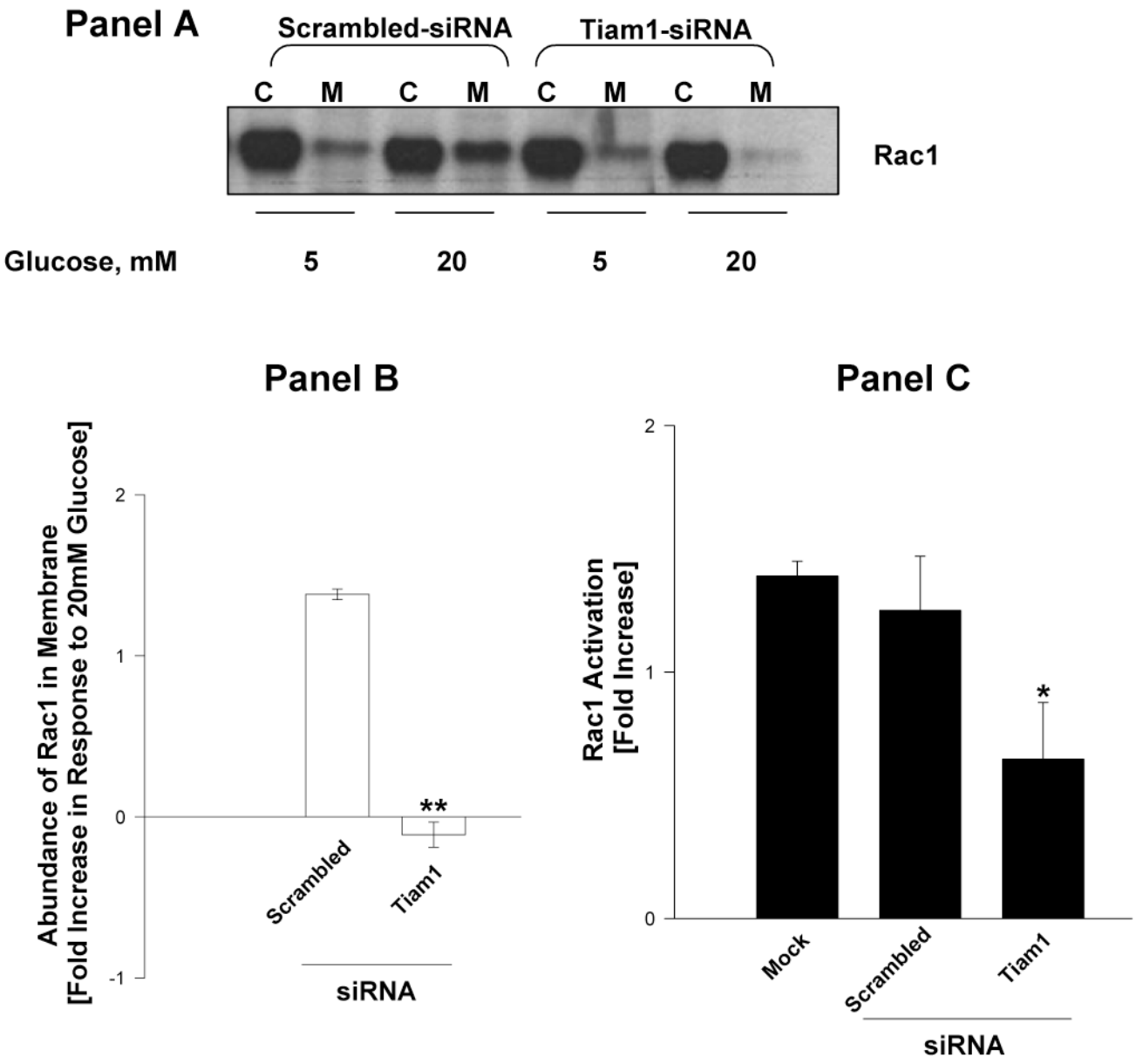

Figure 8. Tiam1 depletion inhibits membrane translocation and activation of Rac1 in pancreatic β-cells

Panel A: Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 24 h, the cells were incubated in the presence of low glucose media overnight. Cells were further incubated in the presence of either low [5 mM] glucose [20 mM] for 30 min at 37°C. Lysates were separated into soluble cytosolic [C] and total membrane pellet [M] fractions by a single step centrifugation method [see Methods]. Proteins from each fraction were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked and incubated with antibody raised against Rac1 [1:1000 dilutions; 1h] followed by incubation with HRP-conjugated secondary antibody. Immune complexes were detected using an ECL kit. A representative blot of three independent experiments yielding similar results is shown here.

Panel B: Intensity of the protein bands was quantitated by densitometry. Data are means ± SEM from three different experiments and expressed as fold increase. **$p < 0.05$ vs. scrambled-siRNA transfected cells challenged with stimulatory glucose concentration alone [20 mM].
Panel C: Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 24 h, the cells were further incubated in the presence of low glucose media overnight. Cells were further incubated in the presence of either low [5 mM] or [20 mM] glucose for 30 min at 37°C. The relative amounts of activated Rac1 [i.e., Rac-GTP] were determined by a pull-down assay [see Methods]. The proteins thus obtained were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, then probed for Rac1. Immune complexes were identified using an ECL kit as described in Methods. Intensity of the protein bands was quantitated by densitometry. Data are means ± SEM from three different experiments and expressed as fold increase. *$p < 0.05$ vs. mock or scrambled-siRNA transfected cells challenged with stimulatory concentrations of glucose [20 mM].

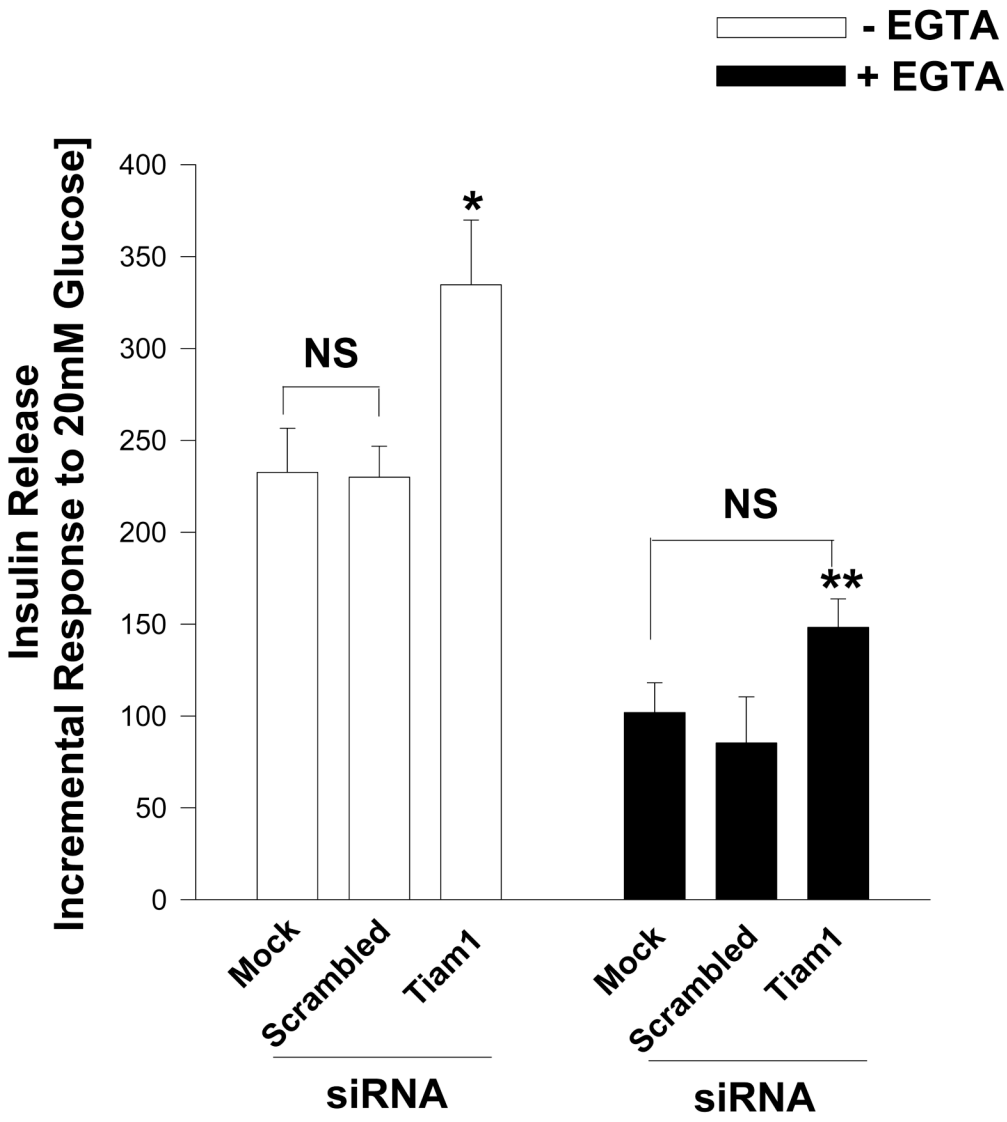

Figure 9. Potentiation of GSIS in Tiam1 depleted pancreatic β-cells is sensitive to extracellular calcium

Pancreatic β-cells were grown at 60–70% confluence and were transiently transfected with 100 nM of siRNA targeted against Tiam1, scrambled-siRNA or mock using HiPerfect transfection reagent [Qiagen, Valencia, CA]. After 24 h, the cells were further incubated in the presence of low glucose media overnight. Cells were further incubated in the presence of either low [5 mM] or [20 mM] glucose media containing 1mM EGTA. Insulin released into the medium was quantitated by ELISA. Data are expressed as incremental response to 20 mM glucose and are means ± SEM from three independent experiments. * represents $p < 0.05$ vs. mock or scrambled-siRNA transfected cells, ** represents $p < 0.05$ vs. Tiam1 transfected cells and NS = Non significant.