

NFAT Regulates Insulin Gene Promoter Activity in Response to Synergistic Pathways Induced by Glucose and Glucagon-Like Peptide-1

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Currently there is intense interest to define the mechanism of action of glucagon-like peptide-1 (GLP-1) in regulating β -cell function, including insulin gene transcription. In this study, GLP-1 (100 nmol/l), in the presence of glucose (11 mmol/l), induced a ~71-fold increase in insulin gene promoter activity in INS-1 pancreatic β -cells, an effect that was an order of magnitude larger than with either stimulant alone. The response to GLP-1 was mimicked by forskolin and largely inhibited by the protein kinase A (PKA) inhibitors, H89 and myristoylated PKI(14–22) amide, indicating partial mediation via a cAMP/PKA pathway. Significantly, the actions of both GLP-1 and forskolin were abolished by the selective Ca^{2+} /calmodulin-dependent phosphatase 2B (calcineurin) inhibitor, FK506, as well as by the chelation of intracellular Ca^{2+} by BAPTA (bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate). Glucose and GLP-1 also synergistically activated NFAT (nuclear factor of activated T-cells)-mediated transcription from a minimal promoter construct containing tandem NFAT consensus sequences. Furthermore, two-point base pair mutations in any of the three identified NFAT sites within the rat insulin I promoter resulted in a significant reduction in the combined effect of glucose and GLP-1. These data suggest that the synergistic action of glucose and GLP-1 to promote insulin gene transcription is mediated through NFAT via PKA- and calcineurin-dependent pathways in pancreatic β -cells. *Diabetes* 51:691–698, 2002

The rate of insulin gene transcription in pancreatic β -cells is regulated by a complex integration of signals derived from nutrients, hormones, and neurotransmitters (1–5). Intracellular calcium ($[\text{Ca}^{2+}]_i$) appears to be an important mediator of this process (3,6–10). We have previously identified NFAT (nuclear factor of activated T-cells) as a key regulator of insulin gene transcription in pancreatic β -cells that is

activated by the calcium/calmodulin-dependent protein phosphatase 2B (calcineurin) in response to increased $[\text{Ca}^{2+}]_i$ (10). NFAT binds to three distinct NFAT elements within the rat I insulin promoter and activates insulin gene transcription. Two signaling pathways arising from glucose metabolism converge to activate NFAT-mediated insulin gene transcription. One pathway results from the direct effect of increased $[\text{Ca}^{2+}]_i$, which activates calcineurin, and in turn, it upregulates insulin gene transcription via NFAT. Glucose metabolism activates this pathway by means of increasing $[\text{Ca}^{2+}]_i$ via L-type voltage-dependent calcium channels (VDCCs) by affecting the electrical activity of the cell. The second pathway also involves glucose metabolism but appears to be driven by glucose-derived factors that target insulin gene transcription independently of $[\text{Ca}^{2+}]_i$.

Glucose metabolism is central to the regulation of β -cell function (11), and GLP-1 is a potent hormonal incretin that has been shown to markedly enhance the stimulatory effects of glucose on β -cells (12,13). For example, GLP-1 potentiates glucose-induced insulin exocytosis. It also increases insulin production by enhancing glucose-induced insulin gene expression. Both glucose and GLP-1 stimulate insulin promoter activity, stabilize proinsulin mRNA, and increase proinsulin biosynthesis (14). It has also been demonstrated that GLP-1 is capable of restoring glucose responsiveness to islets and β -cell lines that have become insensitive to glucose (15,16). Moreover, it has been found that GLP-1 promotes β -cell proliferation and differentiation (17,18). In parallel to these observations in the insulin-producing INS-1 β -cell line, GLP-1 synergizes with glucose to activate expression of immediate-early response genes coding for transcription factors implicated in cell proliferation and differentiation, such as *c-fos*, *c-jun*, *junB*, *zif-268*, and *nur-77* (5,19).

GLP-1 displays pleiotropic effects on the β -cell that are correspondingly supported via the activation of multiple intracellular pathways. The principal consequence of GLP-1 action on the β -cell is to elevate cAMP, although GLP-1 also triggers phosphoinositide 3-kinase (PI 3-kinase) (20). Mechanistically, it is known that GLP-1 increases cyclic AMP in pancreatic β -cells by its action on the Gs-coupled GLP-1 receptor, which in turn, activates cAMP-dependent protein kinase A (PKA) (21). cAMP and PKA have been found to augment glucose-induced intracellular Ca^{2+} -signaling in β -cells by modulating L-type VDCCs (22–25). Moreover, it has also been shown that cAMP has a direct effect on releasing Ca^{2+} from intracel-

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AKAP, A-kinase anchoring proteins; ATF, activating transcription factor; BAPTA, bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate; $[\text{Ca}^{2+}]_i$, intracellular calcium; CAT, chloramphenicol acyltransferase; CRE, cAMP response element; GLP, glucagon-like peptide; NFAT, nuclear factor of activated T-cells; PI, phosphoinositide; PKA, protein kinase A; VDCC, voltage-dependent calcium channel.



FIG. 1. NFAT elements within the rat I insulin gene promoter: relevant position of NFAT elements with respect to other known *cis*-acting elements (in boxes) within the -410 rat I insulin gene promoter. The three NFAT elements (1NFAT, 2NFAT, and 3NFAT) are indicated above the promoter along with the respective NFAT DNA-binding sequences. The underlined base pairs indicate targeted mutations within the NFAT consensus motifs that were used in the promoter-reporter assays to determine the relative functional contribution of each NFAT element to the rInsI promoter in response to glucose and GLP-1.

lular stores in β -cells (26–28). Thus, cAMP is an important regulator of PKA and calcium metabolism in β -cells, and it appears that PKA and calcium contribute to the incretin effect of GLP-1 on β -cells in the presence of glucose (23).

It was hypothesized that glucose and GLP-1 provide factors that synergistically enhance insulin promoter activity via NFAT. This was based on the observation that the calcium-responsive 2NFAT element within the rat I insulin promoter is insensitive to glucose or GLP-1 activation alone but, in contrast, is responsive to the costimulatory effect of these secretagogues in INS-1 β -cells. In the current study, we identify contributing signaling pathways derived by glucose and GLP-1 that play a role in activating NFAT-mediated insulin gene transcription and determine the relative contribution of each of the three NFAT elements within the rat I insulin promoter in producing this response.

RESEARCH DESIGN AND METHODS

Materials. INS-1 cells were obtained from Dr. Mark Prentki (University of Montreal, Centre de Recherches du CHUM and Institut du Cancer, PQ, Canada). FK506 was purchased from Calbiochem, La Jolla, CA. Glucagon-like peptide I(7–36) amide was purchased from Sigma, St. Louis, MO. The NFAT promoter-reporter plasmid (NFAT-Luc) was a gift of Dr. Gerald Crabtree, Stanford, CA.

Plasmids and mutagenesis. Site-directed mutagenesis of three NFAT elements identified within the first 410 bp of the rat I insulin gene promoter (1NFAT, 2NFAT, and 3NFAT) (Fig. 1) was performed as previously described (10). In brief, the NFAT core sequence of consensus motif was disrupted by altering two base pairs (5'-GGAAA to 5'-TCAAAA) and cloned into the pGL2-basic mammalian expression vector (Promega, Madison, WI) to create pGL2-1NFATm, pGL2-2NFATm, and pGL2-3NFATm.

Cell transfections and reporter assays. INS-1 cells (between passages 64 and 72) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/l L-glutamine, 50 μ mol/l 2-mercaptoethanol, 1 mmol/l pyruvate, streptomycin (100 μ g/ml), and penicillin (100 units/ml) at 37°C under an atmosphere of 95% air/5% CO₂. The cells were grown to 60–80% confluency in 12-well plates (Falcon) in RPMI 1640 medium and then brought to 2 mmol/l glucose for 6 h before transfection. INS-1 cell transfection was achieved using FuGene-6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's directions. All cells were cotransfected with a control vector (pSV-CAT) for the normalization of transfection efficiency. Eighteen hours after transfection, the cells were stimulated with 100 nmol/l GLP-1 or 10 mmol/l forskolin in either basal (2 mmol/l) or stimulatory (11 mmol/l) glucose. In experiments involving pharmacological treatments, inhibitors (FK506, 10 μ mol/l; H89, 1–50 μ mol/l; myristoylated PKI(14–22) amide, 1–50 μ mol/l; verapamil, 10–100 μ mol/l; or BAPTA [bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate], 10–100 μ mol/l) were added to the medium 2 h before cell stimulation. The cells were harvested 24 h after transfection (6 h after stimulation) by lysis in Reporter Lysis Buffer (Promega). After brief centrifugation (~16,000g, 5 min) to remove cell debris, the supernatant was assayed for luciferase activity with the Luciferase Assay System (Promega) using a TD-20/20 bioluminometer (Turner Designs, Sunnyvale, CA) or chloramphenicol acetyltransferase (CAT) expression by the CAT-enzyme-linked immunosorbent assay (ELISA) method (Roche Molecular Biochemicals). Data expressed on the ordinate of figures represent arbitrary light units normalized against CAT expression determined in the same samples.

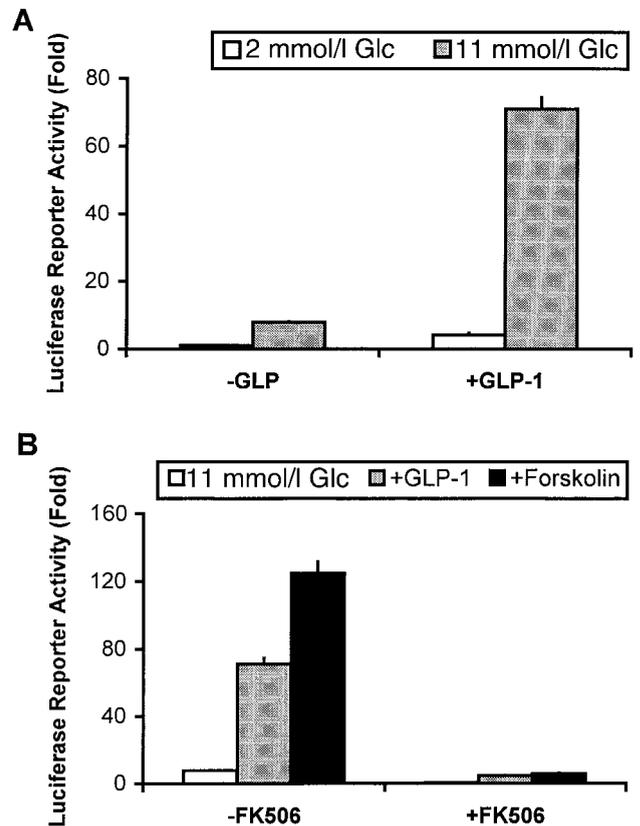


FIG. 2. GLP-1 synergistically enhances the effect of glucose (Glc) on insulin gene transcription and is dependent on calcineurin activity. **A:** INS-1 cells were transfected with pGL2-rInsI and incubated in basal conditions (2 mmol/l glucose) or stimulatory conditions of high glucose (11 mmol/l), GLP-1 (100 nmol/l), or both for 6 h. Lower concentrations of GLP-1 (10–100 nmol/l) were also sufficient to produce a maximal response of the rat I insulin gene promoter (data not shown). **B:** FK506 (10 μ mol/l), selective calcineurin inhibitor, blocks the action of high glucose (11 mmol/l) in the absence (white bars) or the presence of GLP-1 (gray bars) or 10 μ mol/l forskolin (black bars). Data are expressed as a fold increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.

Statistical analysis. Results are expressed as means \pm SE determined from at least three independent experiments, unless otherwise stated. Statistical significance was calculated by one-tailed unpaired Student's *t* test.

RESULTS

GLP-1 synergistically enhances the effect of glucose on insulin gene transcription. Using the experimental regimen of reducing the glucose concentration of RPMI incubation medium to 2 mmol/l for 18 h before INS-1 cell stimulation, the re-establishment of 11 mmol/l glucose (for 6 h) induced a 7.8-fold stimulation of insulin gene promoter-reporter activity (PGL2-rInsI) (Fig. 2A; see also Lawrence et al. [10]). Exposure to 100 nmol/l GLP-1 in the presence of 2 mmol/l glucose induced a significant, but smaller (4.1 ± 0.5 -fold), increase in insulin promoter activity. When added together, GLP-1 in the presence of stimulatory concentrations of glucose promoted a 71.1 ± 3.5 -fold activation of insulin gene promoter activity. This combination thus induced an effect at least an order of magnitude greater than either agent alone, revealing a marked synergistic interaction of glucose and GLP-1 at the level of insulin gene transcription. This observation emphasizes the previously reported ability of GLP-1 to act as a glucose competence factor (15). The dramatic influence

of the combined action of glucose and GLP-1 on insulin gene transcription (appreciably larger than in previous reports) is reasoned to be the consequence of the optimization of the cell incubation protocol, which incorporates a specified period (18 h) of cell incubation in a medium containing low (2 mmol/l) glucose. Presumably, this allows cultured pancreatic β -cells to recuperate from long exposure to media supplemented with stimulatory concentrations of glucose.

GLP-1 mediates insulin gene transcription via cAMP, which requires a Ca^{2+} -dependent pathway involving calcineurin. As mentioned, GLP-1 receptors on the β -cell are coupled to adenylate cyclase via heterotrimeric G proteins, and many of its effects, but not all, are mediated by the generation of cAMP. Not surprisingly, the direct activation of adenylate cyclase, using forskolin, had effects similar to those of GLP-1 on insulin gene promoter activity in the current experimental model. Specifically, forskolin (10 $\mu\text{mol/l}$) induced a 6.0 ± 0.2 -fold and 124.8 ± 7.0 -fold activation of insulin promoter-reporter activity in the presence of 2 and 11 mmol/l glucose, respectively (Fig. 2B), suggesting that the effects of GLP-1 were largely attributable to the elevation of cAMP. Novel to this study, each of these responses was profoundly suppressed by the incubation of INS-1 cells with the calcineurin inhibitor FK506 (10 $\mu\text{mol/l}$), indicating that the signaling required for GLP-1 (and forskolin) induction of insulin gene transcription is dependent on the activation of calcineurin.

Consistent with this implied involvement of calcineurin, the activation of the insulin gene promoter activity by glucose and GLP-1 was found to be dependent on cytosolic $[\text{Ca}^{2+}]_i$. Incubation of INS-1 cells with 100 $\mu\text{mol/l}$ BAPTA, an intracellular Ca^{2+} chelator, prevented insulin promoter activity induced by either glucose (Fig. 3A) or glucose/GLP-1 (Fig. 3B). Verapamil, a selective L-type VDCC-blocker, also inhibited glucose/GLP-1-induced activation of insulin promoter activity, although the effects of this compound were only partial ($\sim 36\%$ at a maximal concentration of 100 $\mu\text{mol/l}$). Similar effects of these Ca^{2+} metabolism antagonists were observed when cells were exposed to forskolin in the presence of glucose. These data indicate that calcium is a requirement for the induction of insulin gene transcription by GLP-1, but further suggest that the elevation of $[\text{Ca}^{2+}]_i$ is derived by means other than voltage-dependent Ca^{2+} influx through L-type Ca^{2+} channels.

Costimulatory effect of glucose and GLP-1 on insulin gene transcription is dependent on PKA. The previous experiments provided evidence that GLP-1 enhancement of cAMP-mediated insulin transcription was dependent on calcineurin. To address signaling via the cAMP-activated PKA, the effects of two selective PKA inhibitors (H89 and PKI) on the activation of the insulin promoter by GLP-1 were assessed. Both H89 and PKI decreased insulin promoter activity (half-maximal inhibitory concentration $[\text{IC}_{50}] < 5 \mu\text{mol}$) in a concentration-dependent manner, reaching a maximal inhibition of $\sim 70\%$ at 10 $\mu\text{mol/l}$ in both cases (Fig. 4A). A similar dose-responsive, but incomplete, effect (80% maximal inhibition) of H89 and PKI was observed for forskolin-induced insulin promoter activity. These data support a conclusion that PKA plays a crucial role in GLP-1-mediated upregulation of insulin gene pro-

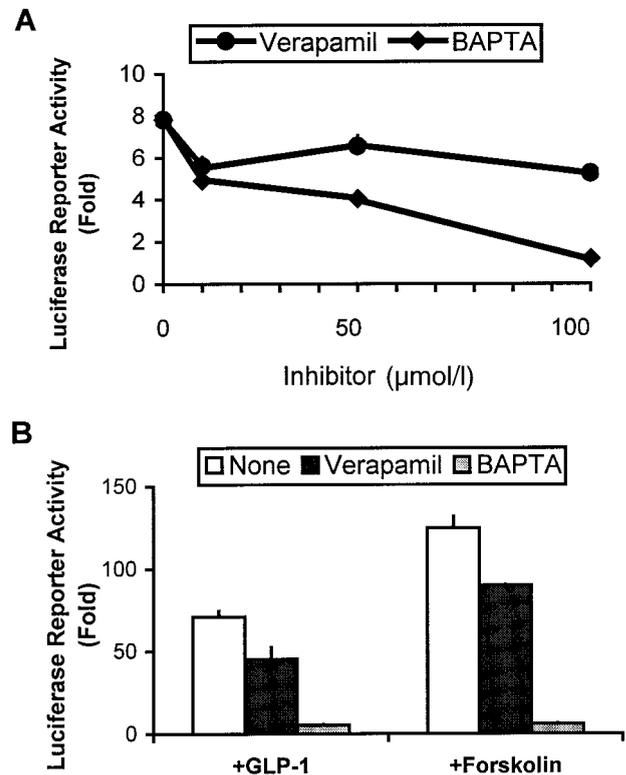


FIG. 3. Calcium is required for the activation of insulin gene transcription by glucose and GLP-1. INS-1 cells were transfected with pGL2-rInsI and treated with calcium inhibitors verapamil or BAPTA 2 h before stimulation. The cells were stimulated with high glucose (11 mmol/l) (A) or both high glucose and 100 nmol/l GLP-1 or 10 $\mu\text{mol/l}$ forskolin (B) for 6 h. Data are expressed as a fold increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.

moter activity, although they also indicate that the activation of this enzyme does not fully account for the synergistic action of GLP-1 or forskolin.

GLP-1 synergistically enhances the effect of glucose on NFAT-mediated gene transcription in β -cells. We have previously shown that calcineurin can activate NFAT in β -cells and that NFAT binds to specific regions on the insulin gene promoter. To address a potential involvement of a similar pathway in the action of GLP-1, INS-1 cells were transfected with a NFAT-reporter construct (NFAT-Luc) in which multiple NFAT-consensus sites were inserted upstream to a minimal promoter (interleukin-2). Just as in the case of the full-length rat I insulin promoter, the costimulatory effect of glucose and GLP-1 on NFAT-reporter activity was greater (13.9 ± 0.5 -fold) than the additive effect of glucose (2.7 ± 0.2 -fold) and GLP-1 (4.1 ± 0.2 -fold) alone (Fig. 5A). These data demonstrate the ability of GLP-1 to activate NFAT, via calcineurin, but further reveal that synergy between glucose and GLP-1 is achieved in part via the activation of this transcription factor. It was also noted that, unlike the insulin promoter, GLP-1 alone had a greater effect on NFAT-reporter activity than high glucose (11 mmol/l), although the significance of this observation is not fully understood.

To determine if the activation of NFAT-mediated transcription in β -cells was mediated by the same signaling mechanisms that activate the full-length rat I insulin promoter, we examined the ability of inhibitors of cal-

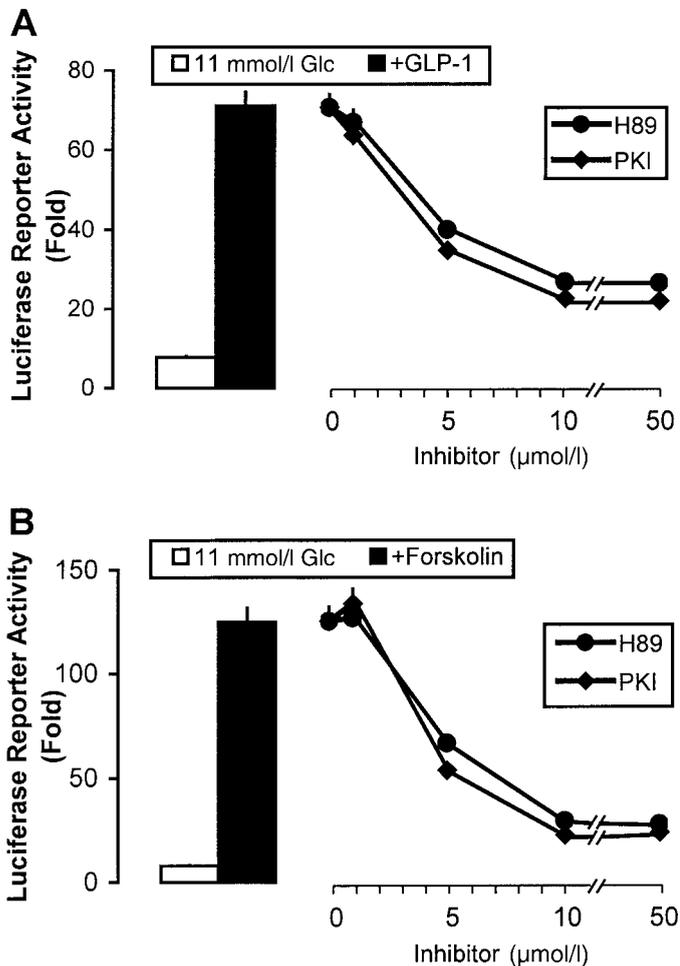


FIG. 4. The synergistic actions of glucose (Glc) and GLP-1 on insulin gene promoter activity are PKA-dependent. Promoter activity of pGL2-rIns1-transfected INS-1 cells in response to the costimulatory effect of high glucose plus 100 nmol/l GLP-1 (A) or high glucose plus 10 μ mol/l forskolin (B) was ablated by increasing concentrations of selective PKA inhibitors H89 (1–50 μ mol/l) and myristoylated PKI (1–50 μ mol/l). Data are expressed as a fold increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.

calcineurin, Ca^{2+} metabolism, and PKA to influence NFAT-Luc-transfected INS-1 cells in the presence of 11 mmol/l glucose and 100 nmol/l GLP-1 (or 10 μ mol/l forskolin). Each of the inhibitors, H89, PKI, FK506, or BAPTA, at the maximally effective concentration, completely blocked NFAT-mediated transcription induced by glucose/GLP-1 and forskolin/glucose combinations (Fig. 5B). This contrasted with the full-length promoter, which still produced a minimal amount of transcriptional activity in the presence of H89 and PKI when costimulated with glucose/GLP-1 (Fig. 4). These results demonstrate that PKA and calcineurin are required for NFAT-mediated gene transcription in β -cells under these conditions.

Effects of NFAT consensus site mutations on the insulin gene promoter. It is significant that each of the NFAT binding site consensus sequences is spatially conserved and distinctly arranged among conserved *cis*-acting elements within 410 bp of the 5'-flanking promoter regions of insulin genes derived from rats, mice, dogs, and humans (with the exception of the 2NFAT element, which is conserved only among rats and mice). To determine the relative contribution of each of the NFAT elements in their

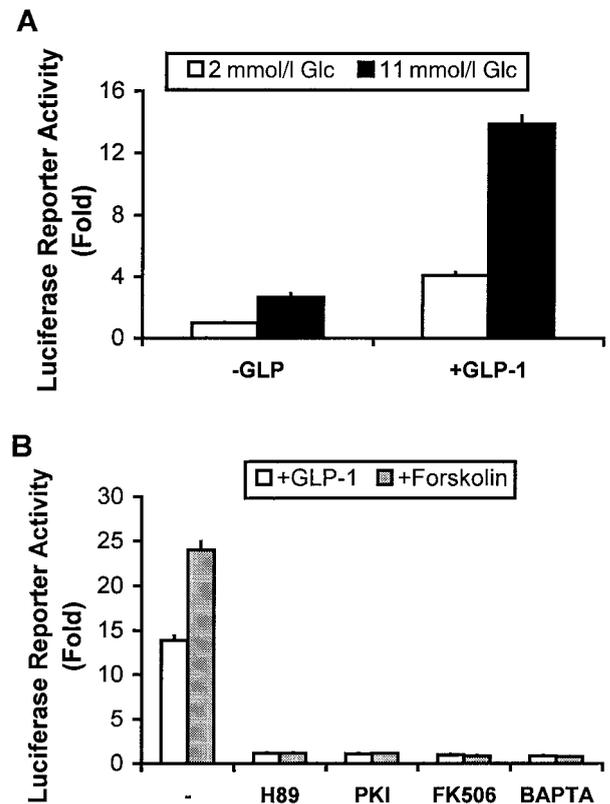


FIG. 5. Glucose (Glc) and GLP-1 synergistically activate NFAT-mediated gene transcription by mechanisms involving PKA, calcineurin, and calcium. A: INS-1 cells were transfected with NFAT-Luc and incubated in basal conditions (2 mmol/l glucose) or stimulatory conditions of high glucose (11 mmol/l), GLP-1 (100 nmol/l), or both. B: Inhibitors H89, PKI, FK506 (10 μ mol/l), or BAPTA (100 μ mol/l) were added for 2 h, before costimulation of INS-1 cells with 11 mmol/l glucose and GLP-1 (or 10 μ mol/l forskolin). Each of the inhibitors completely blocked NFAT promoter-reporter activity in response to combined glucose and GLP-1 stimulation. Data are expressed as a fold increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.

specific response to glucose or GLP-1, each of the NFAT consensus sites within the rat I insulin promoter was mutated by site-directed mutagenesis. A mutation in the NFAT element most proximal to the start site of transcription (1NFAT) significantly reduced the responsiveness of the insulin gene promoter to glucose (~66%; $P < 0.001$) or GLP-1 (~51%; $P < 0.01$) (Fig. 6A). In contrast, a mutated 2NFAT element had little effect (~11% reduction) on glucose-induced insulin promoter activity, and had no significant effect on the induction of insulin promoter activity by GLP-1 (Fig. 6A). A mutated 3NFAT element produced a significant ($P < 0.001$) reduction in both glucose-induced (~24%) and GLP-1-induced (~38%) promoter activity, although not to the extent of the mutated 1NFAT element (Fig. 6A). These data indicate that the NFAT sites within the insulin gene promoter are differentially responsive to glucose and GLP-1. 1NFAT appears to be most important in the activation of insulin promoter activity in response to either glucose or GLP-1 when added independently.

The relative contributions of the NFAT elements became increasingly apparent when the activities of mutant promoters were observed under conditions of costimulation by glucose and GLP-1. A mutation in 1NFAT element

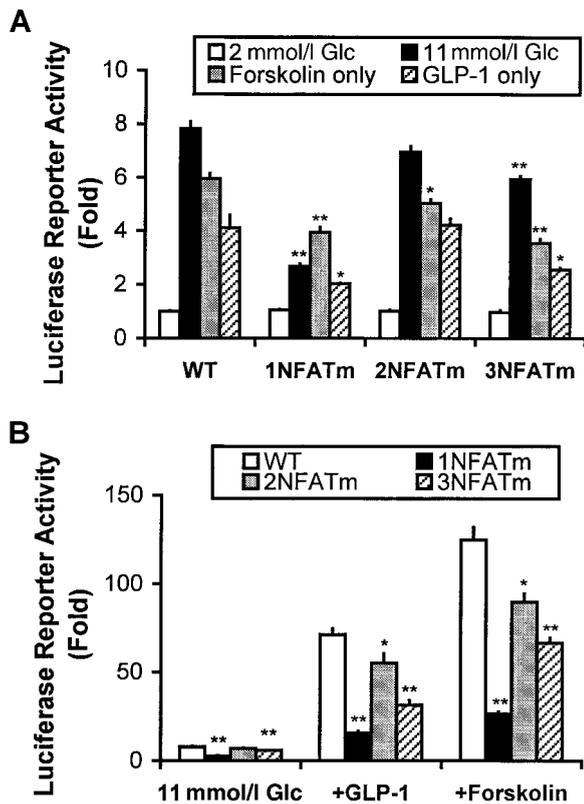


FIG. 6. NFAT elements within the rat I insulin gene promoter are differentially responsive to glucose (Glc) and GLP-1 stimulation. INS-1 cells were transfected with pGL2 reporter vectors harboring rat I insulin gene promoters containing NFAT element site-directed mutations (1-3NFATm) and incubated in basal conditions (2 mmol/l glucose) or stimulatory conditions. **A:** Transfected cells were stimulated with high glucose (11 mmol/l), 100 nmol/l GLP-1, or 10 μ mol/l forskolin. Statistical significance of the data was evaluated by unpaired Student's *t* test: **P* < 0.01 and ***P* < 0.001. **B:** The cells were either treated with high glucose (11 mmol/l) or costimulated with high glucose (11 mmol/l) and 100 nmol/l GLP-1 (or 10 mmol/l forskolin). Statistical significance of the data was evaluated by unpaired Student's *t* test: **P* < 0.05 and ***P* < 0.001. Data are expressed as a fold increase in luciferase activity (normalized to CAT activity) over the wild-type pGL2-rInsI (WT) in the presence of 2 mmol/l glucose. The statistical significance was based on comparisons of the mutant rInsI promoters (1-3NFAT) to WT with respective conditions.

resulted in a dramatic loss (~71%) in the responsiveness of the insulin gene promoter to glucose/GLP-1 (Fig. 6B). The 2NFAT- and 3NFAT-mutated promoters were also significantly (*P* < 0.01 and *P* < 0.001, respectively) reduced in response to glucose and GLP-1, but to a lesser extent (Fig. 6B). The replacement of GLP-1 with forskolin resulted in a similar trend (Fig. 6B). These data show that the synergistic activation of the insulin gene promoter in response to glucose and GLP-1 is largely dependent on intact NFAT elements. They also indicate that the 1NFAT element is critically responsive to both glucose and GLP-1 and that 3NFAT is moderately responsive to these stimulants. In contrast, the 2NFAT element is moderately responsive under conditions of combined effect of glucose and GLP-1, but relatively insensitive to glucose or GLP-1 alone.

DISCUSSION

Glucose is an essential regulator of insulin gene expression from β -cells, and it is likely that glucose is required during any physiological scenario when increased insulin

production is needed (2,11,29-31). In the present study, we identify signaling mechanisms by which GLP-1 synergistically enhances insulin gene transcription in the presence of glucose. Glucose and GLP-1 have similar effects on β -cells, arising from distinct and overlapping signaling pathways. For example, glucose elevates intracellular calcium, but also provides factors to enhance insulin gene transcription by pathways distinct of calcium (10,32,33). GLP-1 increases cAMP and activates PKA, factors both involved in calcium-dependent and -independent pathways in pancreatic β -cells (34-36). Thus, both glucose factors and GLP-1 factors exert multiple effects on the β -cell, and the points of convergence that synergistically upregulate insulin gene transcription are not well understood.

The obvious common effect produced by glucose and GLP-1 is the elevation of $[Ca^{2+}]_i$. Calcineurin, the calcium/calmodulin-dependent protein phosphatase 2B, has been determined to be a major target of calcium in regulating insulin gene transcription in the β -cell (10,37,38). We have recently identified NFAT as a sequential downstream target of calcineurin in the activation of insulin gene transcription by both glucose and depolarizing concentrations of K^+ (10). The current study further suggests that this pathway is also a conduit for the action of GLP-1 to enhance the upregulation of insulin gene expression by glucose. This suggestion is supported by the primary demonstrations that GLP-1-induced activation of insulin gene expression, alone or in combination with glucose, was attenuated by the selective calcineurin inhibitor, FK506, or by the site-directed mutagenesis of identified NFAT binding sites in the insulin gene promoter. Furthermore, GLP-1 was capable of activating NFAT in a calcineurin-dependent manner based on increased activity of a transfected NFAT-reporter construct. Collectively, these data provide further support for an important role of this transcription factor in the physiological regulation of insulin biosynthesis in the β -cell.

GLP-1 heightens $[Ca^{2+}]_i$ in addition to the effect of glucose on β -cells by the potentiation of glucose-induced closure of K_{ATP} channels and subsequent cell depolarization and also by direct influence via PKA-mediated phosphorylation (24,39). Glucose/GLP-1-induced insulin gene transcription is clearly dependent on $[Ca^{2+}]_i$ based on its elimination in the presence of the intracellular Ca^{2+} chelator, BAPTA. However, the action of glucose and GLP-1 was only partially blocked by verapamil. This indicates that whereas L-type calcium channels are involved to some degree, they are not the only source of Ca^{2+} . This is consistent with recent reports that calcium channel type variants are expressed in β -cells (40). In addition to its action on verapamil-sensitive (L-type) VDCCs, GLP-1 can stimulate the opening of Ca^{2+} -activated nonselective cation channels (NSCCs) that are permeant to Ca^{2+} as well as Na^+ (41). Moreover, ω -agatoxin-sensitive (P-type) VDCCs have been identified in β -cells, which contribute to glucose-induced insulin exocytosis (40). Furthermore, GLP-1 has also been reported to elicit fast transient elevations in $[Ca^{2+}]_i$ via Ca^{2+} -induced Ca^{2+} release through type 2 ryanodine receptors on the endoplasmic reticulum (28). These observations may provide insight to other potential sources of calcium that may contribute to glucose/GLP-1-induced insulin gene tran-

scription, although no attempt was made in the current study to directly address the involvement of these specific calcium channels.

The actions of GLP-1 on insulin promoter activity in this study were mimicked closely by forskolin, indicating that they are primarily mediated via the activation of adenylyl cyclase and the generation of cAMP. However, that the effect of forskolin on insulin and NFAT promoter activation was inhibited by FK506 in a manner equal to that of GLP-1 implies that the effects are dependent on calcineurin. The effect of GLP-1, at least at the level of the insulin gene promoter, cannot be completely explained by the activation of PKA, consistent with a previous report (42). That study concluded that there is a direct effect of cAMP on the insulin gene promoter, although the identity of this cAMP receptor was not identified (42). This is in contrast with the complete effectiveness of inhibitors of PKA to prevent GLP-1 and forskolin activation of NFAT. These data suggest that the synergistic action of GLP-1 (or forskolin) and glucose on NFAT is mediated by signaling mechanisms involving both calcineurin and PKA. This could be achieved, for example, at the level of Ca^{2+} signaling via the known effects of PKA to influence Ca^{2+} influx and mobilization via the phosphorylation of L-type Ca^{2+} channels and ryanodine receptor complexes, respectively (39,43). It has been proposed that the synergistic enhancement of increased intracellular calcium by GLP-1 in β -cells is accomplished by PKA-mediated sensitization of type 2 ryanodine receptors to Ca^{2+} invoked by L-type VDCCs, i.e., by Ca^{2+} -induced Ca^{2+} release (CICR) (27,28). Alternatively, another point of convergence could be mediated via the colocalization of calcineurin and PKA to the plasma membrane via interaction with A-kinase anchoring proteins (AKAPs). Indeed, evidence has recently been provided to suggest that the activation of PKA promotes increased activation of calcineurin by promoting its release into the cytoplasmic compartment of the cell, where it would presumably be free to dephosphorylate and activate NFAT. AKAP79 was shown to target PKA to VDCCs in β -cells, and disrupting the targeting function of AKAP prevents both cAMP-mediated elevation of $[Ca^{2+}]_i$ and insulin secretion elicited by GLP-1 (39). Thus, this potentially provides a mechanism whereby GLP-1 facilitates the availability and activation of calcineurin via a mechanism involving the activation of PKA by cAMP.

It is also likely that the interaction of glucose and GLP-1 signaling pathways is mediated by heterologous partnering of critical transcription factors at the level of the insulin gene promoter. Three functional NFAT elements have been identified within the first 410 bp of the rat I insulin promoter. Two of these elements (1NFAT and 3NFAT) are conserved among other known insulin gene promoters of mammals including dogs, mice, and humans. Each NFAT site displays its own distinct responsiveness within the context of the rat I insulin promoter. It is clear that the most proximal NFAT element to the start site of transcription (1NFAT) is most responsive to glucose. The potency of 1NFAT is most likely a result of its embedding within glucose-responsive *cis*-acting elements such as the overlapping A2/C1 binding sites (RIPE3b), which have been reported to bind to glucose-responsive *trans*-acting factors, such as PDX-1, RIPE3b1, and A2-specific factors (44).

PDX-1 (formerly called STF-1, IDX-1, IPF-1, GSF, or IUF-1) is a homeodomain family transcription factor that has been indicated to bind to the A elements of the insulin promoter and interact with transcription factors that bind to neighboring E elements (45). Both glucose and GLP-1 are known to increase PDX-1 activity and expression in β -cells (46). More recently, glucose and GLP-1 have been shown to stimulate PDX-1 nuclear translocation in β -cells by a cAMP/PKA-dependent mechanism (47). The glucose-sensitive RIPE3b1-binding complex, expressed strictly in pancreatic β -cells, is known to bind to specific sequences of both the C1 and A2 elements within the RIPE3b region of the rat II insulin gene promoter and interact with the recently identified A2-specific factors (44). It is important to note that the site-directed two-point mutation of 1NFAT of the rat I insulin gene promoter used in this study was targeted within the core sequence of the NFAT consensus motif, which does not interfere with binding sites of other known transcription factors, such as PDX-1 or CEB (44,45). Curiously, however, the mutated sequence is located near a site that is homologous to base pairs of the rat II insulin gene promoter that are critical for the binding of the RIPE3b and A2-specific binding complexes (44). Given the proximity and similarities in glucose-responsiveness of these elements, NFAT potentially interacts with *trans*-acting factors that bind to these sites during the process of insulin gene promoter regulation. In support of this, we have found that NFAT DNA-binding complexes formed with a probe harboring the 1NFAT element display altered banding patterns in electrophoretic mobility shift assays based on the specific conditions and handling of stimulated islets and INS-1 β -cells (data not shown). Further studies are required to determine these proposed interactions.

The mechanism of NFAT activation is best understood in immune-system cells where NFAT is demonstrated to bind to DNA in complex with members of the basic-region leucine-zipper (bZIP) transcription factors, namely *c-fos/c-jun* (AP-1) (48,49). Based on these observations, it is of interest to identify bZIP transcription factors that potentially interact with NFAT within the insulin gene promoter. An attractive candidate in this respect is the activating transcription factor-2 (ATF-2 or CRE-BP1) of the bZIP family, which in β -cells can be activated by the Ca^{2+} /calmodulin-dependent kinase IV in response to glucose. The calcium-responsive ATF-2 transcription factor binds to four identified cAMP response elements (CREs) of the insulin gene promoter (50). A common theme to the stimulation of cytokine gene transcription by NFAT involves the activation of AP-1 by a lymphokine receptor simultaneously with elevated calcium (48). In a scenario where CaMKIV phosphorylates ATF-2 and calcineurin dephosphorylates NFAT, calcium would suffice to activate both interacting partners in β -cells. Alternatively, both calcineurin and PKA are known to directly activate CRE binding in pancreatic β -cells (3). Thus, a role of calcineurin and PKA in insulin gene transcription may involve the activation of a bZIP member or other required co-activator to induce the insulin promoter. Furthermore, the 1NFAT element itself contains an inverted and overlapping functional bZIP element (CEB/P), which can act as a repressor during prolonged exposure of β -cells to high

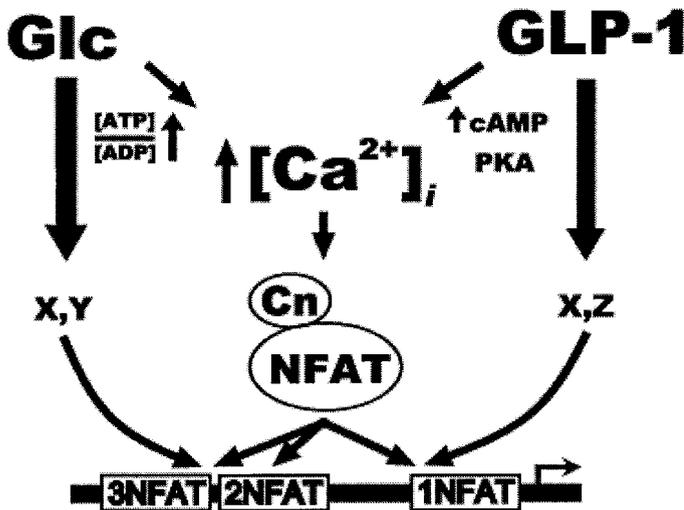


FIG. 7. A simplified schematic of signaling pathways that synergistically activate the rat I insulin gene promoter via NFAT-mediated transcription. Three major pathways that regulate NFAT-mediated insulin gene transcription are derived by 1) glucose factors, 2) GLP-1 factors, and 3) elevation of $[Ca^{2+}]_i$. The first pathway begins with glucose metabolism. The second pathway is largely dependent on PKA. The third pathway is influenced by both the glucose and GLP-1 pathways and is absolutely required. The combination of all three pathways provides a synergistic effect on insulin gene transcription. The synergistic effect is regulated, in part, at the promoter level. Glc, glucose; Cn, calcineurin. X = common factors activated by both glucose and GLP-1; Y = factors activated by glucose; Z = factors activated by GLP-1.

glucose conditions (45). A model for heterologous partnering between NFAT elements within the insulin gene promoter and other transcription factors of the β -cell is yet to be determined.

In any case, it is likely that interactions between NFAT and other *cis*-acting elements within the insulin promoter are required to produce a maximal stimulatory response. This codependency of given factors likely contributes to the synergistic action of glucose and GLP-1 at the insulin gene promoter, such that the individual effect of each stimulator on insulin promoter activity appears to be insignificant in comparison to the extent of activation observed during costimulation. We have recently shown that the 2NFAT element is calcium-responsive and that blocking the calcineurin pathway prevents its activation. However, a mutation of the 2NFAT element does not appreciably affect insulin gene transcription induced by either glucose or GLP-1. This is a unique characteristic of the 2NFAT element with respect to 1NFAT and 3NFAT, which are responsive to either glucose or GLP-1 alone. In contrast, the costimulatory effect of glucose and GLP-1 was significantly ($P < 0.05$) blunted by a 2NFAT mutation. These observations indicate synergism at the promoter level. Hence, glucose and GLP-1 contribute factors that enhance the calcium-responsive NFAT-pathway in addition to the effect that they have on elevating $[Ca^{2+}]_i$ in β -cells. The current study provides further evidence to support this in that the effect of glucose/GLP-1 signaling on isolated NFAT elements in β -cells is completely dependent on PKA, whereas glucose/GLP-1 exerts effects on the full-length promoter (rInsI) that are partially PKA-independent. Furthermore, the fact that the NFAT elements within the insulin gene promoter respond differentially to stimulatory conditions of glucose, GLP-1, or glucose/GLP-1, in

itself, indicates that other transcription factors are modifying the effect of NFAT on insulin gene transcription (or vice versa).

In summary, glucose and GLP1 synergistically upregulate the insulin gene promoter by pathways that converge to enhance NFAT-mediated insulin gene transcription (Fig. 7). This activation requires elevated intracellular calcium and, ultimately, the activation of calcineurin in addition to distinct factors provided by glucose and GLP-1. These factors converge at the level of signal transduction as well as the level of transcription to produce the synergistic effect. The extent to which glucose and GLP-1 synergize to enhance insulin gene transcription supports the notion that combinatory signaling in pancreatic β -cells bears a larger physiological relevance to β -cell function than what is observed by glucose signaling alone.

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