Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic beta cells

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Abstract

We showed previously that ERK1/2 were activated by glucose and amino acids in the pancreatic beta cells. Here we examine and compare signaling events that are necessary for ERK1/2 activation by glucose and other stimuli in beta cells. We find that agents that interrupt Ca\(^{2+}\) signaling by a variety of mechanisms interfere with glucose- and glucagon-like peptide (GLP-1)-stimulated ERK1/2 activity. In particular, calmodulin antagonists and FK506 and cyclosporin, immunosuppressants that inhibit the calcium-dependent phosphatase calcineurin, suppress ERK1/2 activation by both glucose and GLP-1. Ca\(^{2+}\) signaling from intracellular stores is also essential for ERK1/2 activation, as thapsigargin blocks ERK activation by glucose or GLP-1. The glucose-sensitive mechanism is distinct from that used by phorbol ester or insulin to stimulate ERK1/2, but shares common features with that used by GLP-1.
Introduction

Insulin is produced by beta cells in the pancreatic islets of Langerhans in mammals. It is the key hormone that promotes the utilization and storage of glucose. Glucose, on the other hand, is the most important regulator of the secretion and biosynthesis of insulin by beta cells, creating a deceptively simple primary loop controlling sugar metabolism. Signals from Ca$^{2+}$, inositol phospholipids, and cAMP are believed to mediate glucose effects on beta cells, but detailed knowledge of the pathways that control beta cell function is limited (1-6). Van Obberghen and colleagues were the first to show that glucose activates the mitogen-activated protein (MAP) kinases ERK1 and ERK2 in islet-derived cells (7). We and others have confirmed this finding (8-12). MAP kinases, also known as extracellular-signal regulated protein kinases (ERKs), are components of kinase cascades important for transmitting extracellular information to coordinate cellular responses. MAP kinases have been implicated in many physiological events ranging from cellular proliferation and differentiation to cell survival (13;14).

Glucose over its normal physiological concentration range increases the activity of ERK1/2 in pancreatic beta cell lines and intact islets (7;8;10). Glucose metabolism is required for ERK1/2 activation (8), as it is for insulin secretion. Glucose regulation of ERK1/2 has been reported in adipocytes, for example, which also have some capacity for glucose-sensing (15), but in few other cell types. Potentiators of insulin secretion, including forskolin, glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) which promote cAMP synthesis, potentiate ERK activation, leading to the view that ERK1/2 perform functions that depend on the glucose-sensing machinery of beta cells (8;12;16).

Exposure of INS-1 cells to KCl induces Ca$^{2+}$ uptake and ERK activation (8). Inclusion of EDTA or EGTA in the medium blocks activation of ERKs by glucose in INS-1 cells and in islets. Experiments with chelators and with artificially low glucose suggest that ERKs are activated to a small but significant extent even at sub-threshold glucose concentrations, because the activity
in the presence of chelators or at 0-1 mM glucose is lower than activity at 2.8-3 mM glucose in the absence of chelators (8). As confirmation that agents that stimulate insulin secretion via an effect on \( Ca^{2+} \) influx also activate ERKs, effects of the oral hypoglycemic drugs glyburide and tolbutamide, which cause closure of the ATP-dependent potassium channel in the beta cell plasma membrane, were also examined (8;10). Treatment of INS-1 cells with 10 \( \mu \)M glyburide or 100 \( \mu \)M tolbutamide for 2 h increased immunoreactive insulin in the medium by 2-fold in the absence of glucose and also caused a discernible increase in ERK activity. Finally, blockers of L-type \( Ca^{2+} \) channels interfere with glucose-induced ERK1/2 activation, also suggesting the importance of \( Ca^{2+} \) influx on this kinase pathway (10).

Efforts to elucidate the mechanism of ERK1/2 activation have suggested a role for several signaling molecules as intermediates in the pathway. Notable among these is protein kinase C (PKC), which is implicated in one report but discounted by others (11;17). PKC causes activation of ERK1/2 downstream of G protein-coupled receptors that act through Gq and is also involved in prolonged activation caused by some other ligands (18;19). Likewise, glucose induces tyrosine phosphorylation, but its role in ERK1/2 activation by glucose is controversial (11;17;20).

In this study we have examined regulation of ERK1/2 by several extracellular cues, including glucose, GLP-1, and insulin. Our goals were to determine to what extent these agents shared common mechanisms for ERK1/2 activation and to begin to define essential components of the pathways. Our studies reveal that glucose and GLP-1 converge on a common mechanism for ERK1/2 regulation that is distinct from that used by insulin. The mechanism we propose has significant differences from those previously suggested to control ERK1/2 in beta cells; we find a dependence on the natural release of \( Ca^{2+} \) from intracellular stores and a sensitivity to inhibition by immunosuppressants that block the calmodulin-regulated
phosphatase calcineurin.

**Methods**

*Materials* – Recombinant adenoviruses were prepared in this laboratory (K52R ERK2) as described (21) or were kind gifts of L. Klesse (MEK1 S217A, MEK1 S317E, S221E, Raf C4B, Raf BXB, G15A H-Ras) (22) and B. Rothermel (myocyte-enriched calcineurin interacting protein (MCIP1)) (23;24). Forskolin, cyclosporin A, and GLP-1 were purchased from Sigma. KN62, KN93, GF109203X, pituitary adenyl cyclase-activating peptide (PACAP), the calmodulin antagonist W7, thapsigargin, dantrolene, 2-aminoethoxy-diphenyl borate (2APB), nifedipine, diazoxide, rapamycin, the Src inhibitor PP2, and wortmannin were purchased from Calbiochem.

*Cells* - INS-1 cells, either from early passages or subclones selected for increased glucose-stimulated insulin secretion kindly provided by Chris Newgard (6), were grown in RPMI 1640 medium containing 10% fetal bovine serum, 0.5 mM Hepes, pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, and 2.5 mM β-mercaptoethanol (25). Cells that were 60-80% confluent were pre-incubated for 1-2 h in Krebs-Ringer-bicarbonate-Hepes (KRKH) with 0 or 2 mM glucose prior to treatment. In the indicated experiments cells were infected with recombinant adenoviruses at multiplicities of infection of 10-100 for 1 h, 24 or 48 h prior to cell treatment. After treatment with the agents indicated in figure legends, the medium was removed and cells were washed with cold phosphate-buffered saline and harvested in 0.2 ml cold lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.2 μg/ml phenylmethylsulfonyl fluoride, 0.1 M NaF, 2 mM Na₃VO₄, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin (7)). After 20 min on ice, supernatants were collected following centrifugation for 10-30 min at 14,000 rpm in an Eppendorf microfuge and were stored at -80°C.
Measurement of ERK1/2 Activity - Equal amounts of lysate proteins (20-40 μg) were resolved in 10% polyacrylamide gels in sodium dodecyl sulfate and subjected to electrophoresis. Proteins were transferred to nitrocellulose at 700 mA for 1.5 h at 4°C. Membranes were incubated in 5% nonfat milk/0.05% Tween-Tris-buffered saline (TBS) for 1 h, then in 1:3000 anti-phosphoERK1/2 antibody (BioSource or Sigma) in 1% nonfat milk/1% bovine serum albumin/0.05% Tween-TBS for 2 h, and finally in 1:5000 anti-rabbit IgG in 1% nonfat milk/1% BSA/0.05% Tween-TBS for 1 h at room temperature. Membranes were washed twice in 0.05% Tween-TBS and twice in TBS. After detection of phosphorylated ERK1/2 bands (43 kD and 41 kD, respectively) by enhanced chemiluminescence and autoradiography, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 60°C, rinsed with 0.05% Tween-TBS, and immunoblotted as above with 1:5000 Y691 anti-ERK1/2 rabbit polyclonal antibody (26).
Results

Upstream components of the glucose-stimulated ERK1/2 pathway in INS-1 cells-- To examine upstream components of the glucose-dependent MAP kinase cascade, we infected INS-1 cells with adenoviruses expressing kinase-defective or interfering mutants of proteins viewed as core components of the ERK1/2 pathway in other cell types (Ras, Raf, MEK1, ERK2) (14;22). Expression of mutants of the core components that have been shown to inhibit ERK1/2 activation in other cell types (13;14), K52R ERK2, S217A MEK1, Raf C4B, and G15A H-Ras, blocked glucose-stimulated ERK1/2 activity (Fig. 1A-C). In these experiments ERK1/2 activity was monitored in cell lysates with antibodies that selectively recognize the doubly phosphorylated, active forms of the kinases. Total ERK1/2 immunoreactivity demonstrated that equal amounts of the two proteins were present in each lane. Consistent with its inhibitory action, the kinase-defective mutant K52R ERK2 reduced phosphorylation of endogenous ERK1/2. We previously showed that MEK1 but not MEK2 was activated by glucose in INS-1 cells (9), consistent with the finding that the phosphorylation-defective mutant S217A MEK1 blocked ERK2 activation by glucose (Fig. 1B). The effects of the inhibitory Ras mutant and the Raf N-terminal fragment support the conclusion that a small GTP binding protein, either a Ras isoform or another GTPase that uses a common guanine nucleotide exchange factor, are required for ERK1/2 activation in INS-1 cells.

Tyrosine kinases often participate in ERK1/2 activation, and glucose has been reported to induce tyrosine phosphorylation in beta cells (15;20). We examined the potential roles of two tyrosine kinases, Src and PYK2, in inducing ERK1/2 activation. We found no evidence of glucose-induced changes in tyrosine phosphorylation of PYK2 in INS-1 cells (data not shown), suggesting that it is not involved. Src inhibitors including PP2 (27) caused a partial but significant reduction in ERK1/2 phosphorylation in response to glucose (Fig. 1D), suggesting a role for a Src-like kinase.
Calmodulin is the calcium-dependent effector required for stimulation of ERK1/2 by glucose in INS-1 cells-- We previously showed extracellular Ca\(^{2+}\) was required for glucose-dependent ERK1/2 activation (8). Thus, we evaluated the effects of inhibitors that block calcium-dependent signaling molecules. First, we tested protein kinase C (PKC) inhibitors (27) because PKC is required in several systems for ERK1/2 activation and it has been suggested that this is also the case in beta cells (11;28). None of the inhibitors tested, including bis-indoylmaleimide (GF109203X), blocked glucose-induced ERK1/2 activity (Fig. 2A), although phorbol ester-stimulated activity was blocked (not shown). On the other hand, W7, a calmodulin antagonist, was a very effective inhibitor of ERK1/2 activation by glucose (Fig. 2B).

To probe the site of action of calmodulin, we tested the involvement of two important calmodulin targets, Ca\(^{2+}\) and calmodulin-dependent protein kinases (CaMKs) and the calmodulin-dependent phosphatase calcineurin (27;29;30). CaMKII and calcineurin have both been linked to insulin secretion from beta cells (31-38). Not only did the CaMK inhibitors KN62 and KN93, which inhibit interaction of the kinases with calmodulin, both suppress glucose-dependent ERK1/2 activation (Fig. 2C), the immunosuppressant FK506, a calcineurin inhibitor, also prevented ERK1/2 activation by glucose (Fig. 2D).

To determine the specificity of action of FK506, we examined the effects of other immunosuppressants. Supporting an involvement of calcineurin, cyclosporin A and FK520, both of which are also calcineurin inhibitors, prevented ERK1/2 activation by glucose (Fig. 3A and not shown). In contrast, the immunosuppressant rapamycin, which targets mTOR not calcineurin, had no effect on glucose-induced ERK1/2 activity (Fig. 3B). Expression of myocyte-enriched calcineurin interacting protein, a calcineurin inhibitory protein (39), also prevented ERK1/2 activation by glucose (Fig. 3C), providing an independent line of evidence implicating calcineurin in ERK1/2 activation by glucose. To determine if calcineurin inhibitors influence activation of ERK1/2 in other systems, HEK293 cells were treated with EGF or NaCl
to stimulate ERK1/2 activity in the presence or absence of cyclosporin A (Fig. 3D). The calcineurin inhibitor had no effect on ERK1/2 activity induced by either stimulus in 293 cells.

_The calmodulin/calcineurin-dependent mechanism is not common to all agents that stimulate ERK1/2 in INS-1 cells_— Because we had clear evidence that calmodulin/calcineurin were required for ERK1/2 activation by glucose, we wished to determine if other agents that activate ERK1/2 also employ a mechanism dependent on these factors. We examined the potential involvement of calmodulin/calcineurin in ERK1/2 activation by phorbol ester which has not generally been found to use these signal transducers (Fig. 4A). Consistent with an independent mechanism of action, phorbol ester-stimulated activity was not affected by calcineurin inhibitors.

Hormones that stimulate adenylyl cyclase including GLP-1 (Fig. 4B,C) and PACAP (not shown) activate ERK1/2 ((12;16;40), this study). Although the extent of activation was usually less than with either glucose or forskolin, increased ERK1/2 phosphorylation was detected within 2-5 min and persisted for at least 10 min with either GLP-1 or PACAP. The effect on kinase activity was greater if 2 mM glucose was present in the medium. Activation of ERK1/2 by GLP-1 was sensitive to inhibition by W7, but like glucose largely insensitive to wortmannin (Fig. 4B). Although activity stimulated by forskolin alone was apparently less sensitive to the phosphatase inhibitors (Fig. 2B and not shown), activity stimulated by glucose plus GLP-1 or glucose plus forskolin was blocked by FK506 (Fig. 4C). In each of these cases, rapamycin did not reduce ERK1/2 activity.

Because insulin stimulates ERK1/2 in many tissues, and the agents above that trigger ERK1/2 also enhance insulin secretion, we wished to determine if induction of ERK1/2 activity by insulin in beta cells uses a calmodulin/calcineurin-dependent mechanism and is involved in their activation by glucose. Insulin and IGF-1 (not shown) stimulated ERK1/2 in INS-1 cells, but to a much reduced extent compared to glucose (Fig. 4D). Interestingly, activation of ERK1/2 by
insulin was not blocked by wortmannin (lower panel), which inhibits phosphatidylinositol-3 kinase (PI-3 kinase) a major mediator of insulin action (41), although activation of Akt was inhibited (not shown). Wortmannin also had little effect on glucose-stimulated ERK1/2 activity (Fig. 4B). Stimulation of ERK1/2 by insulin, in contrast to glucose, was not reduced by W7 or cyclosporin A (Fig. 4D). These findings suggest that glucose and insulin use distinct mechanisms to trigger ERK1/2.

Source of Ca\(^{2+}\) required for glucose-dependent ERK1/2 activation-- Ca\(^{2+}\) was shown to be required in the earliest studies of this response (7;8). To explore this requirement further, we first depolarized cells by exposing them to 25 mM KCl (Fig. 5A). KCl caused a rapid but transient increase in ERK1/2 activity which had returned to control values by 10 min; glucose activated ERK1/2 more following a 30 min incubation in the presence of KCl than in its absence (not shown). Like activation by glucose, activation of ERK1/2 by KCl was blocked by calcineurin inhibitors (Fig. 5A).

We next examined the source of calcium required for ERK1/2 activation. Nifedipine, a blocker of L-type voltage-gated Ca\(^{2+}\) channels (42), partially blocked glucose stimulation of ERK1/2 (Fig. 5B), as was reported (11;12). Similar results were observed with the related blockers, nisoldipine and nitrendipine (not shown). Under no condition was a complete blockade produced with these agents. Diazoxide is a thiazide which maintains ATP-sensitive potassium channels in the open state, and is used therapeutically to inhibit insulin release from insulin-secreting tumors. This agent at millimolar concentrations blocked glucose activation of ERK1/2 (Fig. 5C); high micromolar diazoxide caused a modest reduction in ERK1/2 activity (not shown). These results indicate that Ca\(^{2+}\) influx is important for ERK1/2 activation.

Ca\(^{2+}\) that is necessary to trigger the kinase cascade could be that resulting from the influx of extracellular Ca\(^{2+}\) or that released from intracellular stores. To determine the impact of the release of Ca\(^{2+}\) from intracellular stores on activation of ERK1/2 by glucose, cells were
pretreated with 1 μM thapsigargin for 15 min, a time sufficient for this ATPase inhibitor to exhaust the intracellular Ca\(^{2+}\) pool (43). Thapsigargin itself did not increase ERK1/2 activity following 5, 10 or 15 min of exposure (not shown). However, activation of ERK1/2 by glucose was completely blocked by thapsigargin (Fig. 6A), suggesting that glucose causes release of intracellular Ca\(^{2+}\) to promote ERK1/2 activation. To compare the effects of glucose to membrane depolarization induced by KCl, we determined if the effects of KCl were also blocked by thapsigargin. Thapsigargin significantly reduced but did not completely block ERK1/2 activation by KCl (Fig. 6B), consistent with the idea that the Ca\(^{2+}\) that activates ERK1/2 is the intracellular pool. For comparison, we also examined the effects of thapsigargin on stimulation of ERK1/2 by insulin (Fig. 4D) and GLP-1 (Fig. 6C). No blockade of insulin-increased ERK1/2 activity was detected, but GLP-1-induced activity was inhibited.

Ca\(^{2+}\) influx can induce the release of this pool through ryanodine receptors and glucose-induced production of inositol trisphosphate (IP\(_3\)) may activate IP\(_3\) receptors (44-48). Thus, we tested dantrolene and 2-aminoethoxy-diphenyl borate (2-APB), inhibitors of Ca\(^{2+}\) release mediated by ryanodine and IP\(_3\) receptors respectively (49;50), to seek independent evidence that intracellular stores are required (Fig. 6D). Both blocked ERK1/2 activation by glucose.
Discussion

Glucose causes the rapid and continuous activation of ERK1/2 in beta cells. The effects of GLP-1 and PACAP, while also very rapid, are short-lived. In comparison, ERK1/2 activation by forskolin is slower but prolonged, perhaps due to actions independent of its ability to increase cAMP or to the much greater accumulation of cAMP it elicits than GLP-1. Because drugs that block GLP-1 are variably effective in blocking forskolin, forskolin may bypass some otherwise essential steps, thereby forcing a cAMP-dependent pathway that does not normally occur in pancreatic beta cells in response to hormones that produce cAMP as a second messenger. Our results further suggest that glucose and GLP-1 converge on a common mechanism of action. In contrast, insulin is generally a weaker ERK1/2 stimulus, and clearly regulates ERK1/2 through a different mechanism. Agents that block glucose- and GLP-1-induced ERK1/2 activity, including calmodulin antagonists and thapsigargin, are ineffective in blocking induction of kinase activity by insulin. Glucose-induced secretion of insulin does not require the release of intracellular Ca^{2+} stores (51;52). In addition, FK506 appears to have little effect on insulin secretion within the first few hours of exposure (34;53-55), although it inhibits glucose-stimulated ERK1/2 activation. These observations further support the idea that the control of ERK1/2 activity exerted by glucose has little to do with the autocrine action of insulin on these cells.

The terminal components of the signaling pathway implicated by the use of interfering mutants include several of the usual suspects. Most clear, MEK1 activity is required; pharmacological inhibitors as well as an inhibitory mutant of MEK1 block ERK1/2 activation, consistent with the finding that MEK1 but not MEK2 is responsive to glucose (9). A small G protein, most likely Ras, is also required, based on the inhibitory effects of a dominant-interfering Ras mutant and of an N-terminal fragment of Raf1, which is thought to act by sequestering activated Ras, preventing it from binding to endogenous Raf proteins. The work
of Bos and colleagues suggests that Rap cannot directly activate Raf, in spite of the fact that the Rap effector domain can bind to Raf (56). The inhibitory effect of the Src inhibitor PP2 suggests that a Src family or other tyrosine kinase leads to stimulation of Ras. Glucose-induced tyrosine phosphorylation has been documented in pancreatic beta cells (20). In contrast to previous reports, we find no evidence for a role of conventional isoforms of PKC, which might act upstream of Ras (11;17). Furthermore, phorbol ester stimulation of ERK1/2 is insensitive to inhibition by the immunosuppressants that completely block the action of glucose on the kinases.

Less clear is the MAP kinase kinase kinase (MAP3 kinase) involved in ERK1/2 activation. Kinase-dead MEK1 would probably interfere with any relevant MAP3 kinase, and the inhibitory N-terminal Raf fragment which contains the Ras binding domain most likely blocks the pathway by targeting Ras, not a Raf family member. In numerous experiments we have found inconsistent activation of Raf-1 and even less evidence for B-Raf activation by glucose in INS-1 cells. However, our findings do not yet convince us that glucose or GLP-1 employs a Raf-independent mechanism, as was recently suggested (12). This is not the only setting in which Raf activity has not been well correlated with ERK1/2 activation (57). Aside from the difficulties of the assay itself, two possibilities seem worthy of consideration. First, cAMP may suppress phosphatase activity that normally inactivates MEK1. And second, cAMP may enhance the formation of Raf1-MEK1 complexes. In either case the efficiency of MEK1 activation would be increased, in spite of minimal Raf activation.

Two conclusions about mechanism are straightforward from our findings. First, the essential mediator of ERK1/2 activation by glucose and GLP-1 is Ca$^{2+}$. Inhibiting Ca$^{2+}$ signaling prevents ERK1/2 activation by either type of agent. Epac2, a cAMP-dependent guanine nucleotide exchange factor for Ras family small GTPases (58;59), is present in pancreatic beta cells and may mediate the actions of cAMP, perhaps directly on Ras. However, our findings
suggest that, if this factor is involved, it likely acts upstream not downstream of Ca\textsuperscript{2+}; this conclusion is consistent with a report from Holz and coworkers who suggest that Epac2 activates Ca\textsuperscript{2+} release in beta cells through effects on the related small G protein Rap (59).

Second, an important source of Ca\textsuperscript{2+} for glucose activation of ERK1/2 is the intracellular storage compartment. By blocking a Ca\textsuperscript{2+} ATPase, thapsigargin depletes Ca\textsuperscript{2+} from this pool. Furthermore, dantrolene blocks calcium-induced release from this pool. The impaired release of Ca\textsuperscript{2+} from the storage compartment completely prevents ERK1/2 activation by glucose or GLP-1, strongly suggesting that the intracellular release of Ca\textsuperscript{2+} is an essential part of the mechanism of ERK1/2 activation by these agents.

Given that both glucose and cAMP cause release of intracellular Ca\textsuperscript{2+} from this pool, perhaps via ryanodine receptors (2;44;59;60), Ca\textsuperscript{2+}-stimulated Ca\textsuperscript{2+} release may be the key process on which these agents converge. Inhibition of glucose stimulation of ERK1/2 by dantrolene supports this conclusion. However, 2-APB also blocks glucose activation. This inhibitor also reportedly interferes with Ca\textsuperscript{2+} entry (61); thus, we can only speculate that the IP3 receptor is also involved in this process. These Ca\textsuperscript{2+} release receptors are reportedly regulated by calcineurin, suggesting that the requirement for calcineurin in activating ERK1/2 may come from its effects on Ca\textsuperscript{2+} release (37;38). If this is correct, calcineurin may be viewed as a gatekeeper, rather than acting directly as a participant, in ERK1/2 activation. Some evidence suggests that the Ca\textsuperscript{2+} release pool may be linked directly to the plasma membrane (62). This juxtaposition may facilitate compartmentalized signaling from receptor and channel complexes; if so, that may account for the rapid effects of GLP-1 and PACAP relative to forskolin.

Inhibitor studies have implicated CaMK II, which is known to be activated by glucose in beta cells (31). It may act downstream of the release of intracellular Ca\textsuperscript{2+} stores, as suggested below. The CaMKII inhibitors have been reported to influence not only other CaMKs, but also Ca\textsuperscript{2+} channels (63); thus, the inhibition we observed may have been caused by effects on Ca\textsuperscript{2+}
signaling that is not mediated by CaMKs at all. This remains to be determined.

Because so many glucose-stimulated components have been implicated in multiple ways by inhibitor studies, it has been difficult to define the signaling pathway leading to stimulation of ERK1/2. We can envision two scenarios that might account for the apparent complexity of our findings. One is that ERK1/2 act as a coincidence monitor in this system in that more than one type of signal must be triggered for activation of the cascade by glucose; for example, based on the comments above, calcineurin might provide the coincident signal. A second is that the kinases are activated by a complex, but linear series of events that have not been previously defined for this pathway.

For future investigation and with the many caveats discussed above, we propose the following pathway (Fig. 7) which incorporates all of the signaling molecules implicated by our inhibitor studies. Glucose metabolism is coupled to Ca$^{2+}$ influx which causes Ca$^{2+}$ release from intracellular stores, through ryanodine and perhaps also IP3 receptors (44). GLP-1 through cAMP also induces Ca$^{2+}$ release via Epac2 from intracellular stores (2;59;64). The actions of GLP-1 and glucose converge at this Ca$^{2+}$ release step, and potentiation of the response may occur as a consequence of the mechanisms by which each agent works on Ca$^{2+}$ release. Depending on calcineurin activity Ca$^{2+}$ release will occur or be prevented. Release of intracellular Ca$^{2+}$ in a discrete location is coupled to activation of a CaM kinase family member. The CaM kinase then employs a tyrosine kinase (e.g., EGF receptors (65;66)) to activate the Ras/ERK1/2 cascade. We are currently devising experiments to test the key steps in this pathway.

ERK1/2 are most frequently implicated in cell proliferation programs. Nevertheless, they are highly expressed in terminally differentiated cells including neurons (67). They play a role in long term potentiation and in synaptic modulation, providing a means of storing signaling information on a longer time scale than individual action potentials (68-71). Their actions in
beta cells may be analogous in that they offer a means of integrating the complex and ever changing nutrient and hormonal signals that acutely control insulin secretion to ensure that beta cells maintain their secretory capacity.
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Figure Legends

Figure 1. Dominant negative mutants of Ras and Raf inhibit ERK1/2 activation by glucose. In panels A-C, INS-1 cells were infected with recombinant adenoviruses expressing the indicated proteins. After 48 h, cells were preincubated in KRBH for 2h and either untreated or exposed to 15 mM glucose (Glc) or Glc plus 10 μM Fsk for 30 min. Lysate proteins were immunoblotted as described in Methods. A. Control, G15A Ras (inhibitory), K52R ERK2 (inhibitory). The phosphorylated ERK2 band visible in the lanes from samples expressing K52R ERK2 comes from the overexpressed ERK2 mutant not the endogenous protein. B. Control, MEK1 S217E, S221E (constitutively active), MEK1 S217A (inhibitory) C. Control, Raf-1 C4B (N-terminal fragment lacking the kinase domain), Raf-1 BXB (constitutively active) D. Cells were preincubated in KRBH for 2 h, then 1 μM PP2 was added for 15 min before the addition of Glc for 30 min. Lysate proteins were immunoblotted as in Fig. 1. Representative data are shown. Experiments were repeated a minimum of 2-3 times. In all panels, duplicate lanes show samples from independent replicates.

Figure 2. Calcium targets required for activation of ERK1/2. INS-1 cells were preincubated as in Fig. 1. The inhibitors were added as indicated 15 min prior to stimulation with 15 mM Glc or as indicated. ERK1/2 activities were detected by immunoblotting as in Fig. 1. A. 2 μM GF109203X B. 50 μM W7 followed by Glc, 10 μM Fsk, or Glc plus Fsk. One of 4 similar experiments C. 10 μM KN62 or KN93; D. 100 nM FK506. One of 10 similar experiments. In all panels, duplicate lanes show samples from independent replicates. Panels A and C show one of 2 comparable experiments.

Figure 3. Calcineurin inhibitors selectively prevent ERK1/2 activation by glucose in beta cells. In A-C INS-1 cells were pre-incubated as in Fig. 1 and duplicate lanes show samples from independent replicates. Cells were untreated or pretreated with: A. 10 μM cyclosporin A
(CSA); or B. 1 μM rapamycin (Rap) for 15 min, followed by the addition of 15 mM Glc for 30 min. C. Cells were infected with an adenovirus expressing mCIP or empty virus. After 24 h the cells were preincubated in KRBH for 2 h and then treated with 15 mM Glc for 30 min. D. 293 cells were pre-incubated in serum-free medium for 24 h, treated with 10 μM CSA for 15 min, and then stimulated with either 10 ng/ml EGF for 5 min or 0.5 M NaCl for 15 min. Experiments in A and B were repeated a minimum of 5 times and in C and D twice.

Figure 4. Effects of inhibitors on ERK1/2 activation by hormones in INS-1 cells. ERK1/2 activities were measured as in Fig. 1. A. Cells were exposed to 1 μM FK506 and then stimulated with either Glc for 30 min or 100 nM phorbol ester (PMA) for 15 min. B. (Upper panel) Cells were pretreated with 50 nM wortmannin (wort) for 15 min followed by 15 mM Glc for 30 min. (Lower panel) Cells were pretreated with wortmannin or W7 for 15 min and stimulated with Glp-1 for 5 min. C. Cells in RPMI with serum and 2 mM Glc for 4 h were exposed to 100 nM Rap or 100 nM FK506 for 15 min, and then stimulated with 11 mM Glc plus 30 nM GLP-1 or Glc plus 10 mM Fsk for 15 min. D. (Upper panel) Cells were treated with W7 and stimulated with insulin for 30 min. (Lower panel) Cells were treated with 10 μM CSA, 1 μM wortmannin, or 1 μM thapsigargin (thaps) for 15 min, and then stimulated with insulin for 30 min. Some panels show duplicate lanes, which represent samples from independent replicates. Data shown are representative of 3 or more similar experiments.

Figure 5. Role of calcium influx in ERK1/2 activation. A. INS-1 cells pre-incubated for 4 h in KRBH plus 2 mM Glc were exposed to 30 mM KCl for the indicated times either without or with a 15-min pre-incubation in 100 nM FK520 or rapamycin. B. Cells treated as in Fig. 1 were pre-incubated in 5 μM nifedipine or 100 nM FK506 for 15 min and then stimulated with 15 mM Glc for 30 min. C. Cells treated as in Fig. 1 were incubated with 1 mM diazoxide for 15 min and then stimulated with 15 mM Glc for 30 min. ERK1/2 activities were analyzed as above. In
B, duplicate lanes show samples from independent replicates. Data shown are representative of 3 or more similar experiments.

Figure 6. Activation of ERK1/2 by glucose requires release of intracellular calcium. INS-1 cells pre-incubated as in Fig. 1 were untreated or pre-treated with 1 μM thapsigargin for 15 min (A-C) and then unstimulated or stimulated with: A. 15 mM Glc for 30 min; B. 25 mM KCl for 10 min; C. 1 μM GLP-1 for 5 min; or D. Cells were pre-treated with 50 μM dantrolene or 50 μM 2-ABP for 15 min and then stimulated with 15 mM Glc for 30 min. ERK1/2 activities were analyzed as above. In A and D, duplicate lanes show samples from independent replicates. Data are representative of at least 3 similar experiments.

Figure 7. Model of regulation of ERK1/2 in pancreatic beta cells.
References

15. Bandyopadhyay, G., Sajan, M. P., Kanoh, Y., Standaert, M. L., Burke, T. R. J., Quon, M. J., Reed,


(2002) FASEB J. 16, 1145-1150


67. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D.,


Fig. 3

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Don Arnette, Tara B. Gibson, Michael C. Lawrence, Bridgette January, Khoo Shih, Kathleen McGlynn, Colleen A. Vanderbilt and Melanie H. Cobb

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