Mode of regulation of the extracellular signal-regulated kinases in the pancreatic β -cell line MIN6 and their implication in the regulation of insulin gene transcription

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Physiological concentrations of glucose that lead to Ca^{2+} entry and insulin secretion activate extracellular signal-regulated protein kinases (ERK1 and ERK2) in the MIN6 pancreatic β -cell line. Here we show that this activation is inhibited by the down-regulation of protein kinase C (PKC) and by genistein, an inhibitor of protein tyrosine kinases. In contrast with results obtained in other cell types, neither the epidermal growth factor activity nor the Src family protein tyrosine kinases seem to be involved in the Ca^{2+} -dependent activation of ERKs. Inhibition of tyrosine phosphatases by vanadate leads to the activation of ERKs. As observed in the response to glucose, this activation is dependent on Ca^{2+} entry through L-type voltage-dependent Ca^{2+} channels. Thus the activation of ERKs in response to glucose

depends on PKC and possibly on a tyrosine kinase/tyrosine phosphatase couple. To define the role of ERK activation by glucose we studied the regulation of transcription of the insulin gene. We found that this transcription is regulated in the MIN6 cells in the same range of glucose concentration as in primary islets, and that specific inhibition of mitogen-activated protein kinase kinase, the direct activator of ERK, impaired the response of the insulin gene to glucose. This was observed by analysis of the transfected rat insulin I gene promoter activity and a Northern blot of endogenous insulin mRNA.

Key words: Ca^{2+} , epidermal growth factor receptor, protein kinase C, Src family kinases.

INTRODUCTION

Mitogen-activated protein (MAP) kinases are serine/threonine kinases involved in many regulatory processes of eukaryotic cells. The best studied MAP kinases are the extracellular signal-regulated protein kinases ERK1 and ERK2, which are implicated in mitosis, cell differentiation and specialized responses to changes in the extracellular medium [1,2]. The activation of ERK1 and ERK2 results from phosphorylation by the MAP/ERK kinases, MEK1 and MEK2, which are themselves regulated by several Raf kinase isoforms. The mechanisms of Raf activation are complex and vary with the stimulus and the cellular system [3]. In the best known systems they involve a small GTP-binding protein such as Ras and a protein tyrosine kinase, pp60c-src. Activation of the MAP kinase pathway occurs in response to the initial activation of receptor or non-receptor tyrosine kinases, the inhibition of protein tyrosine phosphatases or the activation of protein kinase C (PKC) [4]. ERKs respond to growth factors and cytokines and also to an elevation of the cytoplasmic concentration of Ca^{2+} [5].

Changes in the cytoplasmic concentration of Ca^{2+} also have a pivotal role in the regulation of exocytosis. Pancreatic β -cells secrete insulin in response to an elevation of the concentration of glucose in the blood. After the catabolism of glucose, the ATP-to-ADP ratio increases, leading to the closure of the ATP-dependent K^+ (K_{ATP}) channels and the depolarization of the plasma membrane. The secretion of insulin is in turn activated by an entry of Ca^{2+} into the cells through the L-type voltage-gated Ca^{2+} channels. We have recently shown that this Ca^{2+} entry leads

to the activation of the MAP kinases ERK1 and ERK2 in the pancreatic β -cell line MIN6 [6]; these cells secrete insulin in response to physiological concentrations of glucose [7].

Insulin biosynthesis is regulated at the levels of both translation and transcription, but short-term regulation seems to occur mainly at the translational level [8]. We have previously shown that the inhibition of the activation of ERKs does not impair the secretion of insulin in short-term (1 h) experiments and that ERKs are rapidly translocated into the nucleus after stimulation by glucose [6], suggesting their implication in transcriptional regulation. A potential target for this regulation is the insulin gene itself.

The aim of this study was to characterize further the mechanisms of the activation of ERKs in response to glucose, and to determine whether ERKs have a role in the regulation of insulin gene transcription.

EXPERIMENTAL

Number of experiments

All the results presented here were obtained from at least three independent experiments.

Cell culture and stimulation

The MIN6 cells were cultured as described previously [7], in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 15% (v/v) heat-

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; IUF1, insulin upstream factor; K50, 50 mM extracellular K^+ ; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; PKC, protein kinase C; TBST, Tris-buffered saline containing Tween 20.

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inactivated fetal calf serum. Before treatment with reagents, the cells were washed twice in the incubation buffer (IB), which contained 124 mM NaCl, 5.6 mM KCl, 2.5 mM CaCl_2 , 2.5 mM MgCl_2 , 20 mM Hepes, pH 7.0, and 2.8 mM glucose. Before stimulation the cells were incubated for 1 h in IB at 37 °C. Stimulation with the various reagents was performed at 37 °C and stopped at different times by removal of the buffer and immediate cell lysis on ice. In Ca^{2+} -free experiments, after preincubation, the cells were incubated for 15 min in Ca^{2+} -free IB containing 1 mM EGTA and stimulated in the same buffer. The stimulation with elevated extracellular K^+ concentration was done by increasing the K^+ concentration to 50 mM and decreasing the Na^+ concentration to 79.6 mM to maintain isotonicity (K50): 79.6 mM NaCl/50 mM KCl/2.5 mM CaCl_2 /2.5 mM MgCl_2 /20 mM Hepes (pH 7.0)/2.8 mM glucose.

Preparation of protein extracts

Cells were lysed in cold lysis buffer containing 1 % (v/v) Nonidet P40, 0.1 % SDS, 158 mM NaCl, 10 mM Tris, pH 7.8, 1 mM PMSF and 1 mM Na_3VO_4 . Protein extracts were prepared at 4 °C. After lysis, the lysates were collected and centrifuged for 15 min at 12000 *g*. The pellets were discarded. With γ -globulin as a standard the protein concentration in the supernatants was determined by the Bradford microassay method (Bio-Rad). The protein extracts were used immediately for immunoblotting analysis.

Immunoblotting

Equal amounts of protein (40–50 μg) were separated by SDS/PAGE [10 % (w/v) gel] and transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell). The nitrocellulose membrane was incubated for 1 h with 1 % (w/v) gelatin in Tris-buffered saline [10 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 0.05 % (v/v) Tween 20 (TBST) to block non-specific binding. The anti-(active ERK) antibody (dilution 1:20000; Promega) used recognizes the doubly phosphorylated MAP kinases ERK1 and ERK2 on the activating sites corresponding to residues 183 and 185 in ERK2. The membrane was washed extensively with TBST and incubated for 1 h with horseradish peroxidase-conjugated goat anti-(rabbit IgG) (dilution 1:10000; Dako), then washed with TBST. The bands were detected by enhanced chemiluminescence (ECL[®]; Amersham). Before reprobing with the polyclonal anti-ERK2 C-14 antibody (dilution 1:1000; sc154; Santa Cruz) (this antibody recognizes ERK2 and also, weakly, ERK1), the anti-(active ERK) antibody was washed away by incubation for 2 h at 60 °C in 100 mM 2-mercaptoethanol/2 % (w/v) SDS/62.5 mM Tris/HCl (pH 6.8), followed by extensive washing in water and incubation (15 min, twice) in TBST. Polyclonal anti-PKC antibody against the ϵ isoform (Gibco BRL) was used at 1:2000 dilution. Monoclonal antibodies against the α , β I and γ isoforms (Seikagaku) were used at 1:200 dilution. Polyclonal anti-(PKC β II) antibody (Santa Cruz Biotechnology) was used at 1:1000 dilution.

Measurement of cytosolic concentration of Ca^{2+}

MIN6 cells were grown on glass coverslips for 3 days before the experiments. The cells were loaded with 5 μM fura-2 acetoxymethyl ester during a 60 min incubation at 37 °C in a Hepes-buffered medium, pH 7.4, containing 124 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 1.8 mM glucose and 0.1 % BSA. After being loaded, the cells were washed with the same buffer and placed at the bottom of an open perfusion chamber built for microscopic work. The chamber was placed on the stage

of an inverted fluorescence microscope (Diaphot; Nikon) and maintained at 37 °C in a climate box. A flow rate of 1 ml/min was used during the whole experiment. The time necessary for a complete change of the medium in the chamber was 140 s. This delay was taken into account in the graphical representations. The microscope was equipped with a $\times 40$ fluor oil-immersion objective. A selected area of the cells was excited at 340 and 380 nm alternately (every 2 s) and the fluorescence intensity emitted at 510 nm was measured by using a PhotocanII microfluorimeter (Photon Technology International, Kontron, France). Photodamage of the islet cells was minimized by using the smallest amount of illumination required. Background fluorescence was recorded for both wavelengths in areas void of cells and the results were subtracted from the corresponding measurements of fura-2-loaded islets. The measurements of successive 340 nm/380 nm fluorescence ratios reflect the cytosolic concentration of Ca^{2+} [9,10].

Plasmids

The previously described rINS-I-Cat plasmid [11], corresponding to a portion of the rat insulin I gene promoter and extending from the transcription start point to –410 bp, was a gift from H. Edlund (University of Umeå, Umeå, Sweden). RSV-LacZ expressing the β -galactosidase gene from *Escherichia coli* under the control of the Rous sarcoma virus early promoter was a gift from I. Sansal (Institut Gustave Roussy, Paris, France). DNA was prepared by using the Qiagen maxiprep method and quantified spectrophotometrically.

Transfections

MIN6 cells at 50 % confluence were transfected with 4 μg of DNA with the use of a cationic lipid method (Clonfectin; Clontech). For each condition in one experiment, three plates were transfected simultaneously and the lysates were pooled to assay the expression of chloramphenicol acetyltransferase (CAT) and β -galactosidase. Cells were incubated for 7 h with the lipid–DNA complex in DMEM containing 25 mM glucose, then washed with PBS and incubated for 36 h in DMEM containing 0.7 % BSA and different concentrations of glucose in the presence or absence of 95 μM PD98059, as indicated in the Figure legends.

CAT and β -galactosidase assays

The expression of CAT was measured with a Boehringer Mannheim ELISA kit in accordance with the manufacturer's protocol. The cells were lysed in the lysis buffer provided and the extract was used for CAT and β -galactosidase assays. After the determination of protein concentration, the same amount of protein (150 μg) was used for each condition and the measurements were done in duplicate. The RSV-LacZ plasmid was used as a control for transfection efficiency. The β -galactosidase assay was done by the method of Hall et al. [12]. The results presented were obtained from five independent experiments and normalized for β -galactosidase expression.

Northern blot analysis of insulin mRNA

Cells were rinsed with PBS and scraped in denaturing solution [4 M guanidine thiocyanate/25 mM sodium citrate (pH 7)/0.5 % sarcosyl/0.1 M 2-mercaptoethanol]. RNA was isolated by the method of Chomczynski and Sacchi [13]. Total RNA (10 μg) was separated on a 1.5 % (w/v) agarose/formaldehyde gel and transferred to a nylon hybridization membrane (Hybond N⁺; Amersham Pharmacia Biotech) by electroblotting. The membrane was prehybridized overnight in 50 % (v/v) formamide/

5 × SSC/5 × Denhardt's/50 mM sodium phosphate/0.1 mg/ml salmon sperm DNA/0.1 % SDS at 42 °C, then hybridized for 16 h with a ³²P-labelled Syrian hamster preproinsulin cDNA probe [14] in the same solution. The membrane was washed three times at room temperature in 2 × SSC/0.1 % SDS, then twice at 60 °C in 0.2 × SSC/0.1 % SDS and exposed to X-ray films (Kodak Biomax) for 1 h. Under the hybridization conditions used, the probe hybridized with a single 0.5 kb band from the agarose-gel fractionation of total MIN6 cell RNA. Membranes were stripped for 2 h at 75 °C in 1 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.1 × Denhardt's, then rehybridized with a ³²P-labelled oligonucleotide probe specific for 18 S ribosomal RNA to control for variations in the amount of total RNA.

RESULTS AND DISCUSSION

Implication of PKC in the activation of ERKs by glucose

In a previous study we had observed that ERK2 (p42 MAP kinase) and ERK1 (p44 MAP kinase) are regulated by Ca²⁺ entry through L-type voltage-gated Ca²⁺ channels in MIN6 cells [6]. In particular, a physiological concentration of glucose results in the rapid activation and nuclear translocation of ERKs [6]. Inhibition of the activation of ERKs by the PKC inhibitors GF109203-X and RO 31-8220 [6] suggested a role for PKCs. To analyse this point further, we determined whether the activation of ERKs by glucose was still possible after the down-regulation of PKCs by prolonged treatment with PMA. MIN6 cells were incubated for 18 h with 1 μM PMA; as expected, this resulted in a marked inhibition of the activation of ERKs in response to glucose (Figure 1A). In contrast, the rapid and transient Ca²⁺-dependent activation of ERKs that we had observed previously after treatment of the MIN6 cells with a depolarizing concentration of extracellular K⁺ [6] was almost unaltered by treatment with PMA. Therefore one or more PKC isoforms are involved in the activation of ERKs by glucose but not by 50 mM K⁺ (K50).

To determine which of the Ca²⁺-sensitive PKC isoforms expressed in MIN6 cells was down-regulated by PMA, immunoblots were performed before and after 18 h of PMA treatment. The two Ca²⁺-sensitive isoforms PKC βII (80 kDa) and PKC α (80 kDa) were fully down-regulated by PMA; the other Ca²⁺-sensitive PKC isoforms described, βI and γ, were not detected in MIN6 cells (Figure 1B) (a hippocampal extract was used as a control for PKC expression [15]). The Ca²⁺-insensitive, PMA-sensitive isoform PKCε (92 kDa) was not down-regulated, illustrating that down-regulation does not occur for all PMA-sensitive isoforms of PKC (Figure 1B). In fact, it has previously been shown that PKCε is less sensitive to down-regulation by PMA than the α and β isoforms [16–18].

Previous studies have shown that the α isoform is translocated to the plasma membrane in response to glucose [19] and we observed in immunofluorescence experiments that the βII isoform was already located at the plasma membrane under non-stimulating conditions (results not shown).

The activation of ERKs by K50 is not affected by the down-regulation of PKC; stimulation of the cells by glucose still leads to a partial activation of ERKs. However, the activation of ERKs in response to glucose or K50 is completely blocked in the presence of the L-type voltage-gated Ca²⁺ channel blocker nifedipine [6]. Given that the two Ca²⁺-sensitive isoforms of PKC are almost completely down-regulated by PMA it is likely that a Ca²⁺-dependent pathway that does not use these PKCs leads to the activation of the ERKs in our system. Alternatively, because the K_{ATP} channels are involved in the response to glucose but not to K50, PKC down-regulation could have decreased their sensitivity to glucose.

Figure 1 Activation of ERKs by glucose depends on PKC

(A) MIN6 cells were pretreated for 18 h with or without 1 μM PMA in DMEM containing 15% (v/v) heat-inactivated fetal calf serum. Before the stimulation the cells were preincubated for 1 h at 37 °C with 2.8 mM glucose. Stimulation was performed at 37 °C by adding either glucose (16.7 mM) or PMA (1 μM) for the indicated durations or with an isotonic buffer containing 50 mM K⁺ and 2.8 mM glucose (K50). Immunoblots were performed with anti-(active ERK) antibody (top two rows of panels) and anti-ERK2 antibody (bottom panels). The two bands revealed by the anti-ERK antibody corresponded to ERK1 and ERK2. Two different exposures of the film are shown for anti-(active ERK) immunoblotting. (B) Upper panels: lysates of unstimulated cells either pretreated or not with 1 μM PMA for 18 h were analysed for the expression of several isoforms of PKC. Lower panels: the expression of the other two Ca²⁺-sensitive PKC isoforms was analysed by immunoblotting with a lysate of rat hippocampus as a control. Hp, hippocampal extract; M6, MIN6 cell extract.

In contrast with the results presented here, we had previously shown that two inhibitors of PKC (GF109203X and RO31-8220) inhibit the activation of ERKs in response to glucose or K50 [6]. We speculate that these inhibitors act on a PKC isoform that is not down-regulated by PMA and that this isoform allows the activation of the ERKs in response to glucose and K50. This PKC might act through the regulation of the voltage-activated Ca²⁺ channels.

Taken together, these results show that PKCs are involved in the regulation of the response of the MAP kinases to glucose but they do not demonstrate that a PKC is involved in the pathway linking Ca²⁺ entry to the activation of the ERKs.

Protein tyrosine kinases are involved in the regulation of ERKs by Ca²⁺

In several cell types the activation of ERKs after an elevation of cytoplasmic Ca²⁺ concentration was found to involve protein

Figure 2 Inhibition of the Ca^{2+} -dependent activation of ERKs by genistein but not by AG1478

(A) Cells were stimulated for 5 min in the presence of the indicated concentrations of glucose (in mM) or depolarized by incubation with 50 mM extracellular K^+ (K50). Genistein was added during the preincubation step. For each stimulus, genistein was used at 0, 10, 25, 50 and 100 μM . (B) Cells were stimulated for 5 min with 5 or 40 ng/ml EGF or with 10 mM glucose. AG1478 at a final concentration of either 100 (+) or 250 nM (+ +) was added 15 min before the stimulation. Immunoblots were performed with anti-(active ERK) antibody (upper panels) and anti-ERK2 antibody (lower panels).

tyrosine kinases [5]. To analyse this point in MIN6 cells we used the protein tyrosine kinase inhibitor genistein. Incubation of the cells for 1 h with genistein inhibited the activation of ERKs by glucose or K50 in a concentration-dependent manner (Figure 2A), indicating that this activation is likely to depend on a protein tyrosine kinase activity.

In PC12 cells, the regulation of ERKs by Ca^{2+} entry was shown to occur through transactivation of the epidermal growth factor (EGF) receptor [20,21]. We therefore investigated whether inhibition of the activation of ERKs by genistein in MIN6 cells could reflect an inhibition of the EGF receptor. Incubation of MIN6 cells with the selective EGF receptor inhibitor AG1478 at 100 nM abolished the activation of ERKs after treatment with EGF but had only a weak effect on their activation by 10 mM glucose, even at 250 nM (Figure 2B). Therefore EGF receptor kinase activity is unlikely to be involved in the activation of ERKs in response to glucose. However, the possibility remains that the EGF receptor is involved in our system without any requirement for its intrinsic kinase activity. Indeed, phosphorylation of the EGF receptor after the activation of G-protein-linked receptors has been shown to lead to the activation of ERK either with or without requirement for its intrinsic activity [22,23].

The protein tyrosine kinases of the Src family, for instance p60c-src and p59-fyn, have also been shown to have a role in the activation of ERKs in response to Ca^{2+} [24,25]. To determine whether they were involved in MIN6 cells, we used two structurally unrelated inhibitors of the Src family kinases: herbimycin A (IC_{50} 0.5 μM *in vivo* [26]) and PP1 (IC_{50} 1 μM *in vivo* [27]). The stimulation of ERKs after treatment of MIN6 cells by glucose was unaltered by preincubation with 2.5 μM herbimycin A for 24 h (Figure 3A) and even resulted in a slight stimulation. Inhibition of the activation of ERKs by glucose was observed with PP1 but only at a high concentration. Furthermore, the

Figure 3 Effect of herbimycin A and PP1 on the activation of ERKs

(A) The cells were incubated for 24 h in DMEM containing 15% (v/v) heat-inactivated fetal calf serum with or without 2.5 μM herbimycin A. Stimulation was performed for 5 or 30 min with 16.7 mM glucose after preincubation in 2.8 mM glucose for 1 h. Abbreviations: G, 16.7 mM glucose; Ctl, 2.8 mM glucose. (B) Cells preincubated for 15 min with increasing concentrations of PP1 (0, 10, 20, 30 and 40 μM) were stimulated with 10 mM glucose or 50 mM K^+ (K50). Immunoblots were performed with anti-(active ERK) antibody (upper panels) and anti-ERK2 antibody (lower panels).

activation of ERKs after stimulation with K50 was not inhibited by PP1 (Figure 3B). Note that p60c-src is very weakly expressed in the MIN6 cells (compared with fibroblasts) and that no activation of either p60c-src or p59fyn was observed in kinase assays realized on immunoprecipitated protein after stimulation by K50 or glucose (results not shown).

Taken together, these results with tyrosine kinase inhibitors suggest that a protein tyrosine kinase distinct from the EGF receptor and from Src family kinases is involved in the activation of ERKs in response to Ca^{2+} .

The K_{ATP} channel and the L-type voltage-gated Ca^{2+} channel are regulated by phosphorylation [28–30], suggesting that genistein could inhibit their activity under our conditions. Indeed, although its effects in pancreatic β -cells are not known, genistein has been shown to inhibit Ca^{2+} currents through L-type channels in other cells [31,32]. We therefore analysed the effect of genistein on the changes in cytoplasmic Ca^{2+} concentration by using the fluorescent probe fura-2. Stimulation of MIN6 cells with glucose (16.7 mM) led to an increase in cytoplasmic Ca^{2+} concentration and to Ca^{2+} spikes (Figure 4A). In the presence of genistein this increase was smaller and the spikes were absent (Figure 4B). With K50 the increase in cytoplasmic Ca^{2+} was greater than with glucose and there were no spikes (Figure 4C). In the presence of genistein the initial peak of Ca^{2+} was unchanged but the subsequent plateau was smaller (Figure 4D). Inhibition of the plateau by genistein was confirmed by including genistein in the perfusion medium when the plateau phase had already been reached (Figure 4E).

These results indicate that a protein tyrosine kinase activity might regulate the Ca^{2+} entry elicited by glucose and K50 in β -

Figure 4 Impairment of the glucose- and K50-induced Ca^{2+} rise by genistein

The cells were incubated for 1 h in a Hepes-buffered medium containing 2.5 mM Ca^{2+} , 1.8 mM glucose and 5 μM fura-2 acetoxymethyl ester either with (**B, D**) or without (**A, C, E**) 100 μM genistein. The fluorescence intensities emitted at 510 nm were measured after alternate excitations at 340 and 380 nm. After a short period of equilibration in 1.8 mM glucose the cells were treated as indicated. The successive 340 nm/380 nm fluorescence ratios reflect the cytosolic concentration of Ca^{2+} . The traces are from one of at least three experiments.

cells. However, it should be noted that genistein at 100 μM completely inhibits the activation of ERKs but only partly inhibits the Ca^{2+} increase in response to glucose. Furthermore, although the stimulation by K50 in the presence of genistein still leads to a Ca^{2+} increase greater than that with glucose alone, it does not result in the activation of ERKs. In addition, activation of ERKs in response to K50 is observed very rapidly (in less than 2 min) [6] and is thus likely to be due to the initial increase in Ca^{2+} , which is unaffected by genistein. This suggests that a protein tyrosine kinase is involved downstream of Ca^{2+} entry in the activation of ERKs after membrane depolarization.

Involvement of protein tyrosine phosphatases in the regulation of ERKs

It has recently been suggested that the inhibition of tyrosine phosphatase has a critical role in the early steps of the activation of growth factor receptors that lead to the activation of ERKs

[33]. The addition of vanadate (an inhibitor of tyrosine phosphatases) to MIN6 cells resulted in the activation of ERKs in the presence of non-stimulating concentrations or even in the absence of glucose (Figure 5A). This activation was concentration-dependent and its onset was slow (Figure 5B). When the cells were incubated with vanadate, either in the absence of extracellular Ca^{2+} or in the presence of nifedipine (a blocker of the L-type voltage-gated Ca^{2+} channels), the activation of ERKs was blocked. This shows that the activation of ERKs by vanadate depends on Ca^{2+} entry through L-type voltage-gated channels. We had shown previously that forskolin, which enhances the secretion of insulin in response to glucose [34], was also able to enhance the activation of ERKs in response to glucose [6]; we found here that forskolin also potentiates the activation of ERKs in response to vanadate (Figure 5B).

The activation of ERKs by vanadate could result from changes in the state of phosphorylation and activity of the ionic channels. In addition, vanadium compounds could inhibit P-type ATPase

Figure 5 Activation of ERKs by vanadate depends on Ca^{2+} entry

(A) MIN6 cells were incubated for 1 h with 1 mM vanadate in the presence of 3, 1.5 or 0 mM glucose. (B) The cells were incubated with 0.1 or 1 mM vanadate for 30 min or for the indicated durations. The stimulations were performed either in the presence of 2.5 mM Ca^{2+} (—), in the absence of extracellular Ca^{2+} and in the presence of 1 mM EGTA (E), in the presence of 50 μM nifedipine (N) or in the presence of 100 μM forskolin (F). (C) Measurement of the cytoplasmic Ca^{2+} concentration was performed as described in the legend to Figure 4. The cells were incubated in the presence of 1.86 mM glucose and 2 mM vanadate for the indicated periods. Final depolarization of the cells was promoted by 50 mM extracellular K^{+} (K50).

Ca^{2+} pumps, which are responsible for the active transport of Ca^{2+} from the cytoplasm to the endoplasmic reticulum [35]. We therefore investigated whether vanadate could by itself increase the cytoplasmic Ca^{2+} concentration. When the cells were incubated in the presence of 2 mM vanadate for 30 min no increase in Ca^{2+} concentration was observed (Figure 5C), although the cells were still able to respond to K50. Moreover, after a 30 min incubation with 1 mM vanadate, carbachol (100 μM) led to an elevation of the cytoplasmic Ca^{2+} concentration, indicating that the endoplasmic reticulum still contained Ca^{2+} (results not shown). We conclude that the basal activity of the L-type Ca^{2+} channels (in the presence of less than 3 mM glucose) is sufficient to obtain a Ca^{2+} -dependent activation of ERKs in response to vanadate.

Taken together, these results suggest that a tyrosine kinase/tyrosine phosphatase pair is involved in the Ca^{2+} -dependent activation of ERKs.

Regulation of the transcription of the insulin gene by ERKs

Using PD98059, a specific inhibitor of the activation of MEK1 and MEK2, we and others have shown that although the activation of ERKs and the regulation of insulin secretion share important characteristics, ERKs do not regulate the secretion of insulin, at least in short-term experiments [6,36]. Because ERKs are translocated to the nucleus in response to glucose and are known to regulate transcription in other systems, we investigated their possible implication in the regulation of the insulin gene. MIN6 cells transfected with a construct containing the -410 to +1 base pair region of the rat insulin I gene promoter were treated with different concentrations of glucose either in the presence or in the absence of a concentration of PD98059 known to inhibit ERK activation completely [6,37]. Whereas others

Table 1 Regulation of the transcription of the insulin gene by ERKs

MIN6 cells were transiently transfected with rINSI CAT plasmid and immediately treated with different concentrations of glucose, either with or without the MEK inhibitor PD98059 (95 μM) in the presence of 0.7% BSA. After 36 h of treatment, the expression of CAT was measured by an ELISA method. The expression in the cells incubated with 3 mM glucose was arbitrarily set to 1. Results are means $\pm$ S.D. for five independent experiments. Statistical analysis with Student's *t* test: $P \leq 0.005$ between cells treated with 3 and 10 mM glucose; $P \leq 0.0005$ between cells treated with 10 mM glucose and 10 mM glucose plus PD98059, and between cells treated with 16 mM glucose and 16 mM glucose plus PD98059; $P \leq 0.00001$ between 3 mM and 16 mM glucose treated cells.

Glucose concentration (mM)	Relative CAT expression	
	DMSO	PD98059
3	1	0.64 $\pm$ 0.16
10	1.67 $\pm$ 0.22	0.88 $\pm$ 0.15
16.7	2.32 $\pm$ 0.12	1.26 $\pm$ 0.27

observed that the sustained activation of ERKs might involve a MEK1- and MEK2-independent pathway not inhibited by PD98059 [38], in our system PD98059 still inhibited the activation of ERKs observed after several hours (results not shown). We found that the rat insulin I gene promoter responds to glucose in MIN6 cells in a concentration-dependent manner, and that PD98059 inhibits this response (Table 1). We then determined whether regulation of the insulin gene by ERKs could be observed at the level of the endogenous insulin mRNA. The cells were incubated for 18 h in 2 mM glucose and then for 36 h with two different concentrations of glucose either in the presence or in the absence of PD98059. Total RNA was extracted and Northern

Figure 6 Regulation of the endogenous expression of insulin mRNA

Northern blot analysis of the expression of the insulin mRNA in MIN6 cells. The cells were cultured in the presence of 2.8 mM glucose for 18 h and then placed in serum-free medium containing 2.8 or 10 mM glucose with or without 95 μ M PD98059. After 36 h, total RNA was extracted and subjected to Northern blot analysis with a preproinsulin cDNA probe (upper panel). The membrane was rehybridized with a probe specific for 18 S ribosomal RNA to control for variations in the amount of total RNA (lower panel).

blot analysis was performed with a preproinsulin cDNA probe. Figure 6 shows that the amount of insulin mRNA was increased after treatment with 10 mM glucose and that this increase was smaller in the presence of PD98059.

These results suggest that ERKs regulate insulin gene expression in response to an elevation of the extracellular glucose concentration. Transcriptional regulation of insulin biosynthesis has been shown to depend on both ubiquitous and β -cell-specific factors [39–41]. The β -cell-specific human factor insulin upstream factor, IUF1 (the analogue of the mouse factor IPF1 and the rat factor STF1/IDX1), which binds to four sites in the human insulin gene promoter (the A boxes), is involved in the transcriptional response to glucose [42]. High concentrations of glucose lead to the phosphorylation of IUF1 by a p38 MAP kinase (SAPK2a) dependent mechanism [43]. This phosphorylation enhances the binding of IUF1 to the insulin promoter. However, because there is no inhibition of the IUF1-regulated transcription by PD98059 in the MIN6 cells [43], the regulation of transcription of the insulin gene reported here is unlikely to be due to phosphorylation of IUF1 by ERKs. In addition, several other sequences of the insulin promoter are responsive to glucose [44]. It will therefore be important to identify the transcription factors that are regulated by the activation of ERKs in β -cells.

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