The mitogen-activated protein kinase pathway in rat islets of Langerhans: studies on the regulation of insulin secretion

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The expression of mitogen-activated protein kinases (MAPKs) and MAPK kinases (MEKs) in rat islets of Langerhans and the involvement of MAPKs in regulated insulin secretion were examined. Two major isoforms of both MEK (45 and 46 kDa) and MAPK (42 and 44 kDa) were detected in rat islets and shown to be localized to insulin-secreting β cells by detection of their expression in the β cell line MIN6. The tyrosine phosphatase inhibitor sodium pervanadate, and, to a lesser extent, the serine/threonine phosphatase inhibitor okadaic acid, stimulated MAPK phosphorylation, as assessed by a shift in its electrophoretic mobility and by increased phosphotyrosine immunoreactivity of immunoprecipitated MAPK. The increase in MAPK phosphorylation stimulated by sodium pervanadate was not coupled to an increase in MAPK activity, but okadaic acid, either alone or in the presence of sodium pervanadate, caused an increase in myelin basic protein phosphorylation by MAPK. Neither okadaic acid nor sodium pervanadate, either individually or combined, stimulated insulin secretion. 4β-phorbol myristate acetate stimulated an increase in phosphorylation of the 42 kDa isoform of MAPK (*erk2*) in human umbilical vein endothelial cells, but neither it nor glucose affected either the phosphorylation state of islet *erk2* or the activities of immunoprecipitated islet MAPKs. These results provide evidence for the presence of a regulated MAPK pathway in adult rat islets, but our data suggest that MAPK activation alone is not a sufficient stimulus for insulin secretion.

INTRODUCTION

Pancreatic β cells secrete insulin in response to elevated levels of circulating glucose, and the secretory response to glucose is modulated by a variety of hormones and neurotransmitters (reviewed in [1]). There is now a substantial body of evidence to suggest that protein kinases play key roles in transducing extracellular signals received by β cells into the final exocytotic release of insulin. So far there has been considerable interest in the involvement of second-messenger-activated protein kinases that phosphorylate substrate proteins on serine and/or threonine residues in β cell stimulus–response coupling pathways. The major serine/threonine kinase families, those activated by Ca^{2+} and calmodulin (CaMK), Ca^{2+} and phospholipids [protein kinase C (PKC)] and cAMP [protein kinase A (PKA)], have been implicated in either the initiation or amplification of insulin secretion (reviewed in [2]), but there is little information about the involvement of another class of serine/threonine kinases, mitogen-activated protein kinases (MAPKs), in the regulation of the insulin secretory process.

The MAPK family is composed of a number of isoforms, most of which have molecular masses in the range 40–45 kDa (reviewed in [3]). As the name suggests, MAPKs are targets for extracellular mitogens such as platelet-derived growth factor and nerve growth factor, and their role in cell proliferation and differentiation is well documented (reviewed in [3,4]). The involvement of MAPKs in the transduction of non-mitogenic signals has received much less attention, but it is now becoming apparent that they may function as intermediaries in stimulus–secretion coupling cascades in a number of fully differentiated cell types (see, for example, [5–8]).

Unlike CaMK, PKC and PKA, whose activities are regulated directly by the generation or increased availability of the appropriate second-messenger molecules, MAPK activities are under the control of an upstream kinase cascade. Immediately proximal to MAPKs are a number of dual-specificity MAPK kinases (MEKs), which phosphorylate MAPKs on both threonine and tyrosine residues. MEKs are themselves phosphorylated and activated by MEK kinases, including raf-1 and MEKK, which lie upstream to the MEKs. Activation of phorbol ester-sensitive isoforms of PKC has been reported to stimulate MAPK phosphorylation and activity in several cell types [9–12]. The precise mechanisms by which PKC activation results in stimulation of MAPK activity are unclear, although there is evidence for both raf-1-dependent [13] and -independent [11,14] mechanisms. Irrespective of the signalling pathways involved, it is possible that activation of MAPK by PKC may be one mechanism by which phorbol esters exert their intracellular effects, one of which is stimulation of the exocytotic release of granule contents from secretory cells.

The observations that MAPKs are involved in secretion, at least in some cell types, and that their activities may be modulated by the conventional serine/threonine kinase, PKC, have led us to examine the expression of MAPKs and MEKs in adult rat islets and whether the phosphorylation state and activity of MAPKs can be correlated with insulin secretory responses to glucose, the major physiological insulin secretagogue, and to 4β-phorbol 12-myristate 13-acetate (PMA), a PKC activator.

Abbreviations used: CaMK, Ca^{2+}/calmodulin-dependent protein kinase; ECL, enhanced chemiluminescence; HUVECs, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; PKA, protein kinase A; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate.

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EXPERIMENTAL

Materials

Collagenase (type XI), BSA (fraction V), PMA, myelin basic protein (from rabbit brain), protein A–Sepharose, PMSF and leupeptin were purchased from Sigma. $CaMK_{290-309}$ was obtained from Calbiochem (Nottingham, U.K.), PKI_{6-22} from Gibco BRL (Paisley, U.K.) and Ro 31-8220 was kindly provided by Dr. Geoff Lawton, Roche Research Centre. Tissue culture media, antibiotics and foetal calf serum were obtained from Gibco BRL (Paisley, U.K.). Polyclonal pan anti-MAPK IgG, monoclonal anti-*erk*2 IgG and monoclonal anti-phosphotyrosine IgG were from Upstate Biotechnology Inc. (New York, U.S.A.), polyclonal anti-MEK IgG was from Affiniti Research Products Ltd. (Nottingham, U.K.) and horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Dako Ltd. Enhanced chemiluminescence (ECL) reagents and [^{125}I] for insulin iodination were from Amersham International, and [γ^{32}P]ATP (3000Ci/mmol) was from DuPont (UK) Ltd. All other reagents were of analytical grade from BDH. Rats (Sprague–Dawley; 200–250 g) were supplied by King's College London Animal Unit. MIN6 β cells [15] were kindly provided by Professor J.-I. Miyazaki and Dr. Y. Oka (University of Tokyo, Japan).

Tissue preparation

Islets of Langerhans were isolated from rat pancreata by collagenase digestion as previously described [16], and were used immediately after isolation for all experiments. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by collagenase digestion using a modification of the method described by Jaffe and co-workers [17], and experiments were performed on passage 2 cells. MIN6 β cells were maintained in culture in DMEM supplemented with 15% (v/v) foetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin, in a humidified atmosphere of 5% CO_2.

Immunological detection of islet MEKs and MAPKs

Groups of 300–400 freshly isolated islets, or 10^6 β cells (MIN6 insulinoma), were disrupted by sonication in gel electrophoresis sample buffer [18]; proteins were resolved on 10% (w/v) polyacrylamide gels and transferred to 0.2 μm nitrocellulose membranes with a Pharmacia semi-dry electrophoretic blotting unit (0.8 mA/cm^2, 3 h). Membranes were blocked overnight in PBS supplemented with 0.05% (v/v) Tween 20 and 5% (w/v) non-fat dry milk and then incubated for 2 h in the presence of either affinity-purified polyclonal anti-MEK or anti-MAPK antibodies (both antibodies used at 1 μg/ml). Membranes were washed (4×15 min) in PBS/0.05% (v/v) Tween-20 and, for detection of both MAPKs and MEKs, incubated with peroxidase-conjugated goat anti-rabbit IgGs (125 ng/ml) for 1 h. Binding of the goat anti-rabbit IgGs to the MEK or MAPK antibodies was detected by ECL according to the manufacturer's instructions. The MEK antibody was directed against a 13.6 kDa N-terminal region of MEKs 1 and 2 (45 and 46 kDa) and the MAPK antibody was reactive to a 38-residue peptide common to a number of MAPK isoforms (pan anti-MAPK antibody).

Phosphorylation of MAPK

Groups of 300–400 islets were incubated in the presence of stimulators of insulin secretion (PMA or glucose) or of inhibitors of protein phosphatases (okadaic acid and/or sodium pervanadate) for 5–30 min and disrupted by sonication in electrophoresis sample buffer. The phosphorylation state of MAPK(s) was assessed both by phosphotyrosine immunoblotting and by mobility shift assays. For determination of tyrosine phosphorylation, MAPKs were immunoprecipitated from the islet extracts by using the pan anti-MAPK antibody, subjected to PAGE, transferred to nitrocellulose and probed with a monoclonal anti-phosphotyrosine antibody, and immunoreactive proteins were detected by ECL. The phosphorylation state of the 42 kDa isoform of MAPK (*erk*2) was determined after fractionation of islet and HUVEC proteins by PAGE under conditions where the 14.3, 21.5 and 30 kDa rainbow molecular mass markers were run off the separating gel. Proteins were transferred to nitrocellulose and probed with an antibody directed against *erk*2. Immunoreactive proteins were revealed by ECL or by a colorimetric method using 4-chloro-1-naphthol as a horeseradish peroxidase substrate.

Measurement of MAPK activity

Groups of 250 islets were incubated (15 min, 37 °C) in the presence of insulin secretagogues or phosphatase inhibitors, sonicated in lysis buffer (20 mM Tris buffer, pH 7.4, supplemented with 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 50 μg/ml leupeptin, 500 μM vanadate, 1 μM okadaic acid, 250 μM NaF, 20 μM TLCK, 20 μM E64) and the MAPK activity of the islet extracts was assessed by measuring the transfer of ^{32}P from [γ-^{32}P]ATP (final specific radioactivity 4 Ci/mmol) to myelin basic protein (MBP) in the presence of inhibitors of CaMK, PKC and PKA (10 μM $CaMK_{290-309}$, 5 μM Ro 31-8220 and 1 μM PKI_{6-22}). Proteins were separated by PAGE, and MBP phosphorylation was detected by autoradiography. In some experiments MAPKs were immunoprecipitated from islet extracts by using the pan anti-MAPK antibody and Protein A–Sepharose. MBP phosphorylation by the Protein A-immune complex was performed in the presence of [γ-^{32}P]ATP (4 Ci/mmol), but in the absence of inhibitors of CaMK, PKA and PKC activities, and the extent of phosphorylation was determined by autoradiography.

Insulin secretion

Groups of three islets were incubated (30 min at 37 °C) in 200 μl of a physiological salt solution [19] supplemented with glucose or phosphatase inhibitors. Insulin content of the supernatant was determined by radioimmunoassay [18].

RESULTS

Identification of MEK and MAPK isoforms in islets of Langerhans and β cells

MAPK is considered to be a ubiquitous enzyme, but a necessary prerequisite to examination of the involvement of the MAPK family in stimulus–secretion coupling in islets of Langerhans was an examination of the expression of both MAPK and its upstream activator, MEK, in islet extracts. Polyclonal antisera directed against consensus sequences within the MEK and MAPK families detected two major isoforms of MEK and two of MAPK (Figure 1). The MEK isoforms had molecular masses of 46 and 45 kDa and are likely to correspond to MEKs 2 and 1 respectively, against which the antibody was directed. A minor MEK isoform with a molecular mass of approx. 44 kDa was also detected, but the identity of this form is not known. It can be seen from Figure 1 (lower left) that islets clearly expressed two isoforms of MAPK, with molecular masses of 44 kDa (*erk1*) and 42 kDa (*erk2*). In one experiment (out of four) a third immunoreactive protein

(approx. 43 kDa), of unknown identity, was also detected. Islets are a heterogeneous population of cells in which β cells account for about 80 % of the total cell mass. It is therefore likely that the MEK and MAPK immunoreactivities detected in islet extracts are localized to β cells, and immunoprobing of pure β cell populations (MIN6 cell line) indicated that this was so (Figure 1, right).

Effect of phosphatase inhibitors and insulin secretagogues on MAPK phosphorylation

The presence of both MAPKs and MEKs in islets and in β cells (Figure 1) indicated that there may exist within islets appropriate intracellular pathways leading to the activation of MEKs and MAPKs in response to insulin secretagogues. Initially, the effects of phosphoprotein phosphatase inhibitors on the tyrosine phosphorylation state of immunoprecipitated islet MAPKs were examined. Groups of 400 islets were incubated in the presence or absence of sodium pervanadate and/or okadaic acid, MAPKs were immunoprecipitated, fractionated by PAGE, transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody, after which the membrane was stripped and re-probed with the pan anti-MAPK antibody. It can be seen from Figure 2(a) that MAPKs from islets incubated for 15 min in the absence of any stimulus (2 mM glucose alone) showed virtually no phosphotyrosine immunoreactivity. Exposure of islets to the tyrosine phosphatase inhibitor sodium pervanadate caused a marked increase in tyrosine phosphorylation of one protein in particular, which corresponded to the 42 kDa form of MAPK when compared with the proteins detected when the same membrane was re-probed with the MAPK antibody (Figure 2b). Inhibition of serine/threonine phosphatases by incubation of islets in the presence of okadaic acid for 15 min also caused an increase in phosphorylation of the 42 kDa isoform of MAPK, but this was much less marked than that obtained in the presence of sodium pervanadate alone. The combination of okadaic acid and sodium pervanadate also resulted in preferential phosphorylation of the 42 kDa isoform.

Because the 42 kDa form of MAPK (*erk*2) showed the most pronounced increase in tyrosine phosphorylation in response to pervanadate treatment (Figure 2a), the effects of the insulin secretagogues, glucose and PMA on the phosphorylation state of MAPK were examined by selectively targeting *erk2* with an antibody directed against this isoform alone. In this series of experiments, islets were incubated with appropriate test agents for 5–30 min, islet proteins were resolved by PAGE under mobility shift conditions and *erk2* was detected by Western blotting. Mobility shift assays indicated that islet *erk2* was largely in a dephosphorylated state under non-stimulatory conditions (2 mM glucose; Figure 3a), confirming the results obtained by phosphotyrosine immunoblotting (Figure 2a). Inhibition of tyrosine phosphatases by exposure of islets to 100 μM sodium pervanadate for 5 min caused a decrease in the electrophoretic mobility of *erk2*, indicative of its increased phosphorylation, and the extent of phosphorylation was increased markedly by increasing the exposure time to 15 min (Figure 3a). Incubation of islets in the presence of 10 μM okadaic acid for 15 min also resulted in decreased mobility of *erk2*, but the effect of okadaic acid was much less than that of pervanadate at 15 min and exposure to okadaic acid for 5 min was without effect on *erk2* phosphorylation (Figure 3a). In contrast to the results obtained with the phosphatase inhibitors, exposure neither to 20 mM glucose for 15 min nor to PMA for 15 or 30 min caused an increase in the phosphorylation of *erk2* (Figure 3a). There are several reports (e.g. [10,11,14]) that MAPK phosphorylation is increased by activation of phorbol ester-sensitive isoforms of PKC with PMA, so the lack of effect of PMA in islets was rather unexpected. However, this seemed to be a peculiarity of islets, rather than of experimental protocol, because when HUVECs were exposed to 100 nM PMA for 10 min there was a marked increase in the phosphorylation state of *erk2* (Figure 3b).

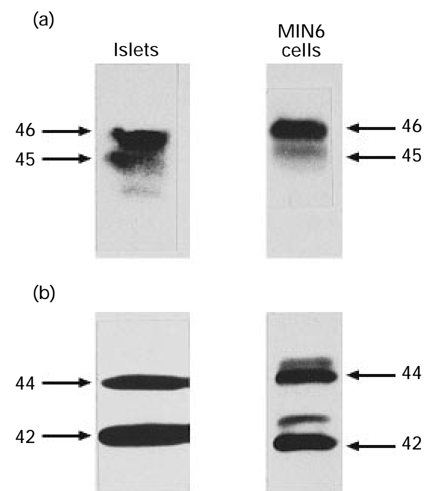

Figure 1 Immunoreactive detection of MEK and MAPK isoforms in islets and β cells

(**a**) Both rat islets of Langerhans (left) and MIN6 β cells (right) expressed two isoforms of MEK, of approximate molecular masses 46 and 45 kDa. (**b**) Rat islets (left) expressed two distinct isoforms of MAPK with molecular masses of 44 and 42 kDa. MIN6 β cells (right) also expressed the 44 and 42 kDa isoforms of MAPK and both isoforms were partly phosphorylated (slower migrating species) in this insulinoma cell line. Similar results were obtained in up to four separate experiments.

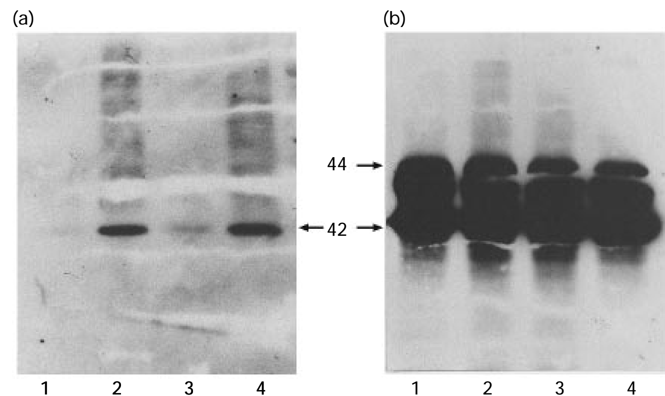

Figure 2 Effect of phosphatase inhibitors on islet MAPK tyrosine phosphorylation

(**a**) In unstimulated islets (2 mM glucose, lane 1) immunoprecipitated MAPK showed little tyrosine phosphorylation, determined with an anti-phosphotyrosine antibody. Incubation of islets for 15 min in the presence of 100 μM sodium pervanadate alone (lane 2) or in combination with 10 μM okadaic acid (lane 4) caused a marked increase in phosphorylation of MAPK on tyrosine residues. Okadaic acid alone (lane 3) marginally increased tyrosine phosphorylation of MAPK. (**b**) Detection of MAPK immunoreactivities by re-probing the same blot with the pan anti-MAPK antibody revealed that it was the 42 kDa form (*erk2*) that showed marked tyrosine phosphorylation after exposure of islets to sodium pervanadate. Results are representative of those obtained in two separate experiments.

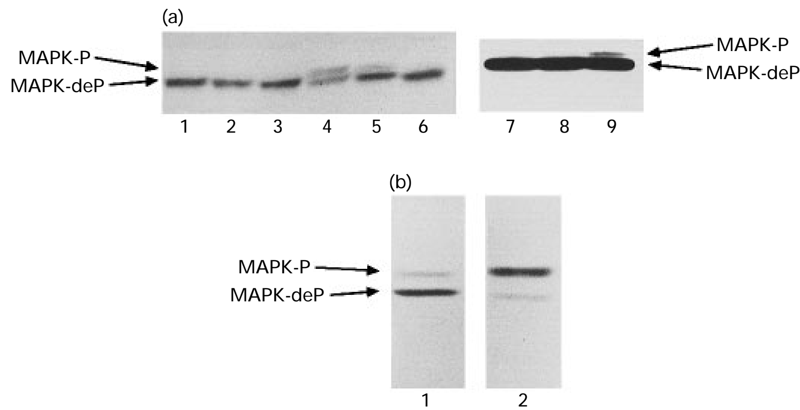

Figure 3 Effect of insulin secretagogues and phosphatase inhibitors on *erk2* phosphorylation

(**a**) In unstimulated islets (2 mM glucose, lanes 1 and 8) the 42 kDa isoform of MAPK (*erk2*) was not phosphorylated (MAPK-deP), as determined by mobility shift assays with the anti-*erk2* antibody. Incubation of islets in the presence of 20 mM glucose for 15 min (lane 2) or 500 nM PMA for 15 min (lane 3) or 30 min (lane 6) did not affect the phosphorylation state of *erk2*. However, stimulation of islets with either 100 μM sodium pervanadate for 5 min (lane 5) or 15 min (lane 4), or with 10μM okadaic acid for 15 min (lane 9) resulted in an increase in *erk2* phosphorylation (MAPK-P). Exposure to 10 μM okadaic acid for 5 min (lane 7) did not cause detectable phosphorylation of *erk2*. (**b**) Although PMA was without effect in islets, treatment of HUVECs with PMA (100 nM for 10 min) resulted in increased phosphorylation of *erk2* (lane 1, unstimulated; lane 2, +PMA). Similar results were obtained in three separate experiments (islets) and in at least four experiments (HUVECs).

Stimulation of islet MAPK activity

The lack of effect of glucose and PMA on islet *erk2* phosphorylation (Figure 3) suggests that these insulin secretagogues do not affect *erk2* activity, because MAPKs require phosphorylation for full activation. However, it is possible that either glucose and PMA stimulate the phosphorylation and activation of the 44 kDa MAPK isoform, or that the results obtained with the mobility shift assay might not correlate directly with the actual phosphorylating capacity of MAPK. We therefore examined the effects of glucose, PMA and phosphatase inhibitors on MAPK activity, assessed by the transfer of ^{32}P from [γ-^{32}P]ATP to the substrate MBP. In initial experiments we exposed islets to test substances for 15 min and determined the ability of islet extracts to phosphorylate MBP. Because MBP is a substrate for several protein kinases, a cocktail of kinase inhibitors was included in the assay buffers to inhibit the activities of CaMK (10 μM $CaMK_{290-309}$), PKC (5 μM Ro 31-8220) and PKA (1 μM PKI_{6-22}). Islets incubated for 15 min in the absence of a stimulus (2 mM glucose) showed little phosphorylation of MBP, and increasing the glucose concentration to 20 mM was without effect (Figure 4a). Exposure of islets to 100 μM pervanadate, a treatment that caused increased phosphorylation of MAPK both in phosphotyrosine immunoblot studies and in mobility shift assays (Figures 2 and 3), did not result in activation of MAPK (Figure 4). Inhibition of serine/threonine phosphatases by exposure of islets to 10 μM okadaic acid, in either the absence or presence of pervanadate, caused a large (4–6-fold) increase in MBP phosphorylation. When the experiments were repeated after immunoprecipitation of islet MAPKs with the pan anti-MAPK antibody, to minimize the involvement of kinases other than those of the MAPK family, qualitatively similar results were obtained. Thus, exposure of islets to 20 mM glucose did not cause increased phosphorylation of MBP by immunoprecipitated MAPKs, pervanadate alone was without significant effect and okadaic acid was stimulatory, in both the absence and presence of pervanadate (Figure 4b). In addition to the lack of effect of glucose, there was no increase in islet MAPK activities after exposure of islets to 500 nM PMA for 15 min (Figure 4b).

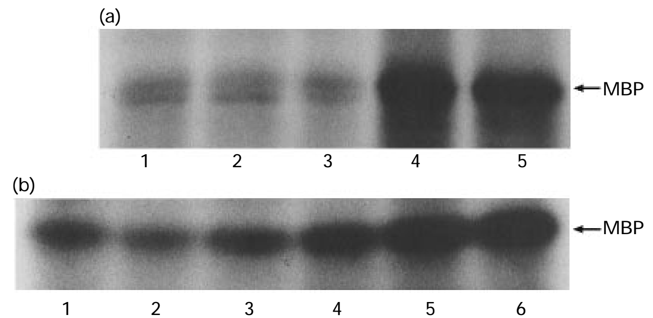

Figure 4 Stimulation of islet MAPK activity

(**a**) Extracts of islets that had been incubated for 15 min in the presence of 2 mM glucose (lane 1) showed little phosphorylation of MBP in the presence of inhibitors of PKC, PKA and CaMK. Similarly, incubation for 15 min in the presence of either 20 mM glucose (lane 2) or 100 μM sodium pervanadate (lane 3) did not result in increased MBP phosphorylation. However, islets that had been incubated in the presence of 10 μM okadaic acid, either alone (lane 4) or in combination with 100 μM sodium pervanadate (lane 5), showed a marked increase in MBP phosphorylation. (**b**) Similar results were obtained when MAPK was immunoprecipitated from islets after 15 min exposure to 20 mM glucose (lane 1), 500 nM PMA (lane 2), 2 mM glucose (lane 3), 100 μM sodium pervanadate (lane 4), 10 μM okadaic acid (lane 5), or 10 μM okadaic acid plus 100μM sodium pervanadate (lane 6). Results are from two separate experiments.

Effect of phosphatase inhibitors on insulin secretion

Measurements of MAPK phosphorylation and activity (Figures 2–4) indicated that pervanadate stimulated MAPK phosphorylation, okadaic acid stimulated both MAPK phosphorylation and its activity, whereas the insulin secretagogues glucose and PMA were without effect on either parameter. To examine further whether there may be a link between MAPK phosphorylation/activation and the exocytotic release of insulin, the effects of sodium pervanadate and okadaic acid on insulin secretion were measured, under conditions (2 mM glucose) where their effects on the phosphorylation state and activity of MAPK had been investigated. It can be seen from Table 1 that exposure

Table 1 Insulin secretory responses to phosphatase inhibitors

Neither inhibition of tyrosine phosphatases with sodium pervanadate (100 μM) nor inhibition of serine/threonine phosphatases with okadaic acid (10 μM) significantly affected unstimulated insulin secretion ($P > 0.1$). Similarly, a combination of the inhibitors did not cause a significant increase in insulin release ($P > 0.1$). Under the same conditions 20 mM glucose caused a significant ($P < 0.001$) stimulation of insulin secretion. Data are means ± S.E.M. for eight or nine individual observations (noted in parentheses), representative of two experiments.

Treatment	Insulin secretion (ng per islet per 30 min)
2 mM glucose	0.10 ± 0.01 (9)
+ 100 μM pervanadate	0.11 ± 0.01 (9)
+ 10 μM okadaic acid	0.13 ± 0.02 (9)
+ 100 μM pervanadate + 10 μM okadaic acid	0.13 ± 0.01 (9)
20 mM glucose	2.04 ± 0.19 (8)

of islets to the phosphatase inhibitors, either individually or combined, had no effect on the basal secretory rate, but islets incubated in the presence of 20 mM glucose showed a 20-fold increase in insulin secretion.

DISCUSSION

There are several reasons why, in principle, members of the MAPK family are ideal candidates for key elements in the stimulus–secretion coupling pathways within pancreatic β cells: MAPK is thought to be ubiquitous; it has been implicated in exocytosis; its activity is modulated by PKA and PKC, kinases that modulate the insulin secretory response; and one intracellular substrate for MAPK is cytosolic phospholipase A_2, which may play a fundamental role in the regulation of insulin release (reviewed in [20]).

Our initial studies indicated that members of both the MAPK family and the upstream regulatory kinase family, MEK, are indeed present in rat islets of Langerhans and in β cells. The predominant isoforms of MEK detected in islets and β cells had molecular masses of 45 and 46 kDa, and those of MAPK were found to be 42 and 44 kDa. The immunoblotting studies suggested that both isoforms of MAPK were phosphorylated in the rapidly dividing MIN6 cells, in contrast with the fully differentiated and essentially non-dividing islet cells, which is perhaps indicative of a role of MAPK in β-cell mitogenesis.

MEKs activate MAPKs by stimulating phosphorylation of a tyrosine and a threonine residue, and MAPKs are dephosphorylated *in vivo* by dual-specificity tyrosine/threonine phosphoprotein phosphatases, which are highly specific for MAPKs. The phosphatases are products of immediate early genes and the loss of MAPK phosphorylation *in vivo* has been shown to coincide with their expression [21,22], but induction of dual-specificity phosphatases seems not to be essential for MAPK inactivation [23,24].

Phosphorylation of MAPK can be detected readily with anti-phosphotyrosine antibodies and by gel mobility shift assays. The first method detects phosphorylation on tyrosine residues alone and the second detects MAPK that is phosphorylated at either or both residues. Mobility shift assays are generally regarded as good indicators of MAPK activity, because physiological activation of dual-specificity MEKs results in phosphorylation of MAPKs on both tyrosine and threonine residues. However, it is possible to obtain a decrease in electrophoretic mobility when only one residue is phosphorylated, and this is not associated with increased MAPK activity [25]. This was observed in the present studies when exposure of islets to sodium pervanadate resulted in marked tyrosine phosphorylation of the 42 kDa isoform of MAPK (*erk2*), decreased electrophoretic mobility of *erk2*, but no increase in MAPK activity. Although pervanadate can inhibit dual-specificity phosphatases [22], it is highly unlikely that this was the mechanism by which it increased *erk2* phosphorylation, because induction of expression of dual-specificity phosphatases is stimulus-dependent; the time-course of synthesis is of the order of 30–60 min [21,22], rather than the 5–15 min time course of the studies with pervanadate; and inhibition of dephosphorylation of both residues would be expected to enhance MAPK activities. It is more likely that pervanadate was inhibiting islet tyrosine-specific phosphatases, which appear to be very active, thus shifting the equilibrium of MAPK phosphorylation/dephosphorylation to the non-physiological situation of being heavily tyrosine-phosphorylated, but threonine-dephosphorylated. MAPKs that are not phosphorylated on threonine residues are unable to exert catalytic activity [25]. The effects of okadaic acid were far more subtle. It caused an enhancement of *erk2* phosphorylation, but this was only a fraction of that stimulated by pervanadate. Again, in the absence of expression of inducible dual-specificity MAPK phosphatases, the effects of okadaic acid are best explained by inhibition of endogenous serine/threonine phosphatases, which play important roles in inactivating the upstream MEKs and MEK kinases. Inhibition of dephosphorylation of either of these kinase families would indirectly lead to increased MAPK phosphorylation (on both tyrosine and threonine residues) and activity, because it would prolong the active state of MEKs and MEK kinases. The effect of okadaic acid on the extent of MAPK tyrosine phosphorylation was much less marked than that of pervanadate, because although MAPK was being phosphorylated by MEK(s) in the presence of okadaic acid the active tyrosine phosphatases were rapidly dephosphorylating it. Nevertheless, the increase in MAPK phosphorylation in response to okadaic acid was sufficient for activation of the enzyme, which resulted in increased phosphorylation of MBP.

Having established that MAPKs and MEKs are present in islets and β cells and that the phosphorylation state and activities of MAPKs are susceptible to regulation by phosphatase inhibitors, we examined the effects of insulin secretagogues on signalling through the MAPK cascade. Activation of phorbol ester-sensitive isoforms of PKC with PMA results in insulin secretion from pancreatic β cells [26], and in other tissues PMA causes MAPK phosphorylation and activation (see, for example, [9–12]). In our studies PMA did not affect islet MAPK (*erk2*) phosphorylation after 5 [27], 15 or 30 min exposure, but in parallel experiments it clearly caused a pronounced increase in the phosphorylation state of endothelial cell *erk2*. These results suggest that although the PKC signalling pathway is linked to the MAPK cascade in many cells, including HUVECs, in islets the activation of phorbol ester-sensitive isoforms of PKC is not a sufficient stimulus for activation of MAPK. This is borne out by our results in which exposure of islets to PMA did not stimulate immunoprecipitated MAPK activities. The lack of effect of PMA on MAPK phosphorylation and activity is unusual, but not unique: similar results have been reported recently in experiments with human platelets [28]. Glucose-induced insulin secretion is dependent, at least in part, on protein phosphorylation, but neither PKA nor phorbol ester-sensitive isoforms of PKC are essential for a secretory response to glucose [2]. Our present results suggest that glucose may also stimulate insulin release independently of activation of MAPKs. Thus, like PMA, glucose had no effect on phosphorylation of *erk2*, and islets incubated in the presence of glucose for 15 min, a time-

course over which the secretory response to glucose is well developed, showed no increase in MAPK activities.

Although glucose and PMA did not activate MAPKs, activation by other agents could potentially result in the stimulation of insulin secretion. In our experiments, inhibition of serine/threonine phosphatases with okadaic acid, under conditions that resulted in a significant increase in MAPK-mediated phosphorylation of MBP, had no effect on insulin secretion. Thus MAPK activation alone appears to be an insufficient stimulus for insulin release from pancreatic β cells.

The results from these experiments do not necessarily rule out an involvement of MAPKs in the regulation of insulin secretion. Our studies have established that two insulin secretagogues, PMA and glucose, do not detectably activate islet MAPKs, and that pharmacological activation of MAPKs with okadaic acid by itself does not result in an insulin secretory response. However, MAPK(s) may play a modulatory role in β cell signal–secretion coupling pathways in response to agonists that act at extracellular receptors, either those associated with tyrosine kinase activity (e.g. growth hormone or prolactin), or those coupled to heterotrimeric GTP-binding proteins (e.g. lysophosphatidic acid). Further detailed investigation is now required to identify whether agonists acting at cell-surface receptors of adult islets stimulate MAPK activities and whether this is involved in the stimulation of insulin secretion by these ligands.

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